High Density Lipoprotein from Patients with Coronary Disease Exerts Altered Endothelial Effects: Role of Proteome Remodeling

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Abstract

Epidemiological studies have strongly suggested that reduced plasma levels of HDL cholesterol are associated with an increased risk of coronary artery disease. Raising HDL Cholesterol is therefore being examined as a potentially important therapeutic strategy. However, recent genetic data and clinical trials have been inconclusive on the beneficial effects of raising HDL cholesterol levels. Clinical trials using the HDL cholesterol raising agents torcetrapib, dalcetrapib and niacin have shown that no significant reduction of cardiovascular events was observed in patients with coronary disease. These recent failures in treatments that raise HDL cholesterol levels have cast doubt on whether HDL plays a truly protective role in cardiovascular disease.

In recent years, various biological functions of HDL have been identified, that may account for the ability of HDL to protect against atherosclerosis. Endothelial dysfunction is suggested to play a critical role in development and progression of atherosclerosis and recent studies have suggested that HDL exerts direct endothelial-protective effects, such as stimulation of endothelial production of the anti-atherogenic molecule nitric oxide, anti-inflammatory, anti-apoptotic, and anti-thrombotic effects. However, accumulating evidence suggests that the vascular effects of HDL may be highly heterogenous and vasoprotective properties of HDL may be impaired in patients with inflammatory or cardiovascular disease. It is therefore essential to elucidate the mechanisms leading to impaired vascular effects of HDL in coronary artery disease and to understand the importance of raising HDL with atheroprotective quality.

The first aim of this thesis was to investigate the effects of HDL isolated from patients with coronary artery disease (CAD) on endothelial function and to characterize the mechanisms leading to impaired vascular effects of HDL in patients with CAD, in particular the effects of HDL on endothelial apoptotic signaling pathways. The second aim of my thesis was to optimize cell-based assays that allow for high-throughput assessment of the endothelial effects of HDL. Finally, in order to elucidate the mechanisms of action of HDL and its altered vascular effects in cardiovascular diseases, there is a need to characterize HDL cargo. The third aim of my thesis was to use targeted proteomics to investigate changes in the protein compositions of HDL that may modulate its function in coronary artery disease.

In order to investigate the effects of HDL on endothelial apoptosis signaling and its alterations in coronary disease, I have isolated HDL from patients with CAD and characterized its mechanisms of action. In contrast to HDL from healthy subjects, HDL isolated from patients with CAD failed to inhibit endothelial cell apoptosis *in vitro* and in apoE-deficient-mice *in vivo*. Instead, HDL isolated from these patients stimulated endothelial pro-apoptotic pathways, in particular p38-MAPK-mediated activation of the pro-apoptotic Bcl-2-protein tBid. This study further suggests that differences in the proteome of HDL from

patients with CAD, in particular reduced HDL-associated clusterin and increased HDL-associated apoC-III, play an important role for altered activation of endothelial anti- and pro-apoptotic signaling pathways.

I have optimized cell-based assays for the assessment of HDL function, in particular the capacity of HDL to inhibit TNF-α-induced VCAM-1 expression, to reduce active caspase-3 activation and to stimulate nitric oxide production. These assays were used to evaluate the impact of cholesteryl ester transfer protein (CETP) inhibitor treatment on the endothelial effects of HDL. Treatment with dalcetrapib failed to restore HDL functions to the level of healthy subjects, which may explain, at least in part, the lack of benefit on endothelial function seen in the dal-VESSEL trial.

Interestingly, despite the failure of torcetrapib in clinical trial resulting in increased cardiovascular mortality, no impairment in HDL functions was observed following torcetrapib treatment in this study. This finding may explain the lack of improvement in the quality of HDL following torcetrapib treatment, however the adverse effects observed in the clinical trial of torcetrapib were likely caused by off-target side effects, which may have been due to direct deleterious actions of torcetrapib on endothelial function, independent of the HDL cholesterol raising effect.

Last but not least, using quantitative proteomics analysis with selected reaction monitoring (SRM), it was observed that HDL from patients with CAD has significantly altered protein compositions as compared to HDL from healthy subjects, further supporting our earlier observations. Notably, significant differences were observed in the HDL proteome between patients with CAD who died from cardiovascular events versus patients who did not develop any events during the entire follow-up study period. This finding may identify potential markers in determining patients at risk of developing future cardiovascular events. More importantly, some of the proteins identified will allow better understanding of the mechanisms of action of HDL and its alterations in cardiovascular disease. Of note, some of the proteins identified to be differentially regulated are not related to lipoprotein metabolism, indicating that HDL physiology and its pathophysiology in CAD is beyond simply regulation of lipid metabolism but also other metabolic processes such as innate immunity and regulation of oxidative stress.

Zusammenfassung

In epidemiologischen Studien waren verringerte Plasmaspiegel des HDL-Cholesterols mit einem erhöhten Risiko für koronare Herzerkrankung (KHK) assoziiert. Daher wird derzeit untersucht, ob die Steigerung der HDL-Cholesterolspiegels im Blut eine erfolgreiche therapeutische Strategie bei KHK darstellt. Jedoch lieferten aktuelle genetische Untersuchungen und klinische Studien widersprüchliche Daten zum Effekt einer Steigerung der HDL-Spiegel. Klinische Studien, bei denen die HDL-steigernden Substanzen Torcetrapib, Dalcetrapib und Niacin eingesetzt wurden, konnte bei Patienten mit KHK keine signifikante Verringerung kardiovaskulärer Ereignisse beobachtet werden. Diese Studienergebnisse führten zu Zweifeln an der postulierten protektiven Wirkung einer HDL-Steigerung bei KHK.

In den vergangenen Jahren wurden verschiedene biologische Funktionen des HDL-Cholesterols identifiziert, welche dessen atheroprotektive Wirksamkeit beeinflussen könnten. Besonders der endothelialen Dysfunktion wird eine wichtige Rolle in der Entstehung und Entwicklung der Atherosklerose zugeschrieben und von aktuellen Studienergebnissen wurde eine endothel-protektive Wirkung des HDL-Cholesterols abgeleitet, beispielsweise die Produktion des anti-atherogenen Stickstoffmonoxids durch Endothelzellen, sowie weitere inflammationshemmende, überlebensfördernde und thrombosehemmende Eigenschaften. Allerdings deuten die Ergebnisse mehrerer Studien darauf hin dass die Eigenschaften des isolierten HDLs sehr heterogen und abhängig vom Gesundheitszustand des Spenders sind. Die gefässprotektiven Eigenschaften des HDL könnten bei Patienten mit inflammatorischen oder kardiovaskulären Erkrankungen verringert sein. Es ist daher wichtig, die Mechanismen zu verstehen, die zu einer Einschränkung der gefässschützenden Eigenschaften des HDL bei Patienten mit KHK führen und die Bedeutung einer Erhöhung speziell von HDL mit gefässschützenden Eigenschaften zu verstehen.

Das Ziel dieser Dissertation war daher zunächst, die Effekte von HDL von Patienten mit KHK auf die Endothelfunktion zu untersuchen und die Mechanismen zu charakterisieren, welche die vaskulären Eigenschaften des HDLs bei Patienten mit KHK einschränken, speziell die Effekte des HDLs auf apoptose-relevante Signalwege in Endothelzellen. Das zweite Ziel war, zellbasierte Tests für die Hochdurchsatz-Analyse der endothelialen Effekte des HDLs zu optimieren. Weiterhin war es wichtig, die Proteinbeladung des HDLs zu charakterisieren, um die Wirkmechanismen des HDLs und seine veränderten vaskulären Effekte bei kardiovaskulären Erkrankungen besser zu verstehen. Das dritte Ziel der Dissertation war daher, die gezielte Proteomanalyse einzusetzen, um Veränderungen in der Proteinzusammensetzung des HDLs zu identifizieren, die dessen Funktion bei Patienten mit KHK verändern könnten.

Für die Charakterisierung der molekularen Effekte des HDL auf zelluläre Signalwege zur Steuerung der endothelialen Apoptose und deren Änderung bei KHK habe ich zunächst HDL von Patienten mit KHK sowie von gesunden Probanden isoliert und dessen molekulare Effekte untersucht. Im Vergleich zu gesundem HDL war HDL von Patienten mit KHK nicht in der Lage, die Apoptose von Endothelzellen *in vitro* und *in vivo* in ApolipoproteinE-defizienten Mäusen zu verhindern. Stattdessen stimulierte das HDL von KHK-Patienten pro-apoptotische Signalwege in Endothelzellen, wie die Aktivierung des pro-apoptotischen Proteins tBid, eines Mitglieds der Bcl-2-Proteinfamilie, durch die Kinase p38-MAPK. Die Studienergebnisse deuten weiterhin darauf hin, dass Unterschiede im Proteom des HDL von KHK-Patienten, einschliesslich einer Verringerung von HDL-assoziiertem Clusterin und einer Erhöhung von HDL-assoziiertem ApoC-III, eine wichtige Rolle bei der Modulierung von endothelialen pro- und anti-apoptotischen Signalwegen spielen.

Ich habe zellbasierte Untersuchungsmethoden der HDL-Funktion für Hochdurchsatzanalysen optimiert, speziell die Bestimmung der Fähigkeit des HDLs, die TNF-α-induzierte Hochregulation der VCAM-1-Expression und die Aktivierung der Caspase-3 zu verringern, sowie die Produktion von Stickstoffmonoxid zu induzieren. Diese Methoden wurden verwendet, um den Einfluss von Inhibitoren des Cholesterylester-Transferproteins (CETP) auf die endothelialen Effekte des HDLs zu testen. Die Behandlung mit Dalcetrapib bei KHK-Patienten konnte die HDL-Funktion nicht auf die Werte des HDLs von gesunden Probanden anheben. Dies könnte, zummindest teilweise, erklären warum in der "Dal-VESSEL"-Studie keine Verbesserung der Endothelfunktion beobachtet werden konnte. Interessant ist, dass wir trotz des Versagens von Torcetrapib in klinischen Studien, bei denen eine erhöhte Mortalität beschrieben wurde, keine Verschlechterung der HDL-Funktion beobachteten. Dies mag erklären, warum keine Verbesserung der HDL-Qualität durch Torcetrapib erreicht werden konnte. Die beobachteten unerwünschten Ereignisse, die in der Torcetrapib-Studie beobachtet wurden, wurden möglicherweise durch Nebenwirkungen des Medikaments hervorgerufen, beispielsweise durch direkte, HDL-unabhängige Effekte des Torcetrapibs auf die Endothelfunktion.

Im letzten Teil der Dissertation konnte mithilfe der quantitativen Proteomanalyse, bei der die Technik „selected reaction monitoring (SRM)" eingesetzt wurde, gefunden werden, dass sich die Proteinzusammensetzung des HDLs von Patienten mit KHK signifikant vom HDL gesunder Probanden unterscheidet. Dies unterstützt zusätzlich unsere früheren Beobachtungen. Von besonderer Bedeutung ist die Identifizierung von Unterschieden in der HDL-Zusammensetzung von Patienten mit KHK, die später an einem kardiovaskulären Ereignis verstarben, im Vergleich zu Patienten, welche während des follow-up-Zeitraums kein Ereignis erlitten hatten. Diese Beobachtung könnte in der Zukunft zur Identifizierung von Markern dienen, um Patienten zu erkennen, die ein erhöhtes Risiko haben, ein kardiovaskuläres Ereignis zu erleiden. Weiterhin ermöglichen einige der identifizierten Proteine ein besseres Verständnis der

Wirkmechanismen des HDLs und seiner Veränderungen bei kardiovaskulären Erkrankungen. In diesem Zusammenhang ist von besonderem Interesse, dass für einige der verändert vorhandenen Proteine bislang keine Rolle im Lipoproteinmetabolismus bekannt war. Dies legt den Schluss nahe, dass die physiologische Rolle des HDL, sowie seine pathophysiologischen Veränderungen bei KHK , sich über die einfache Regulation des Lipidmetabolismus erstreckt und weitere Prozesse, beispielsweise die unspezifische Immunabwehr und die Regulation des oxidativen Stresses beeinflusst.

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Abbreviations

A1AT	alpha-1-antitrypsin
A2MG	alpha-2-macroglobulin
ABCA1	ATP binding casette transporter A1
ABCG1	ATP binding casette transporter G1
ACS	acute coronary syndrome
apoA-I	apolipoprotein A1
apoA-II	apolipoprotein A2
apoC-III	apolipoprotein C-III
APOL1	apolipoprotein L1
AQUA peptide	absolute quantification peptide
Bad	Bcl-2-associated death promoter
Bcl-xL	B-cell lymphoma-extra large
Bid	BH3 interacting domain death agonist
CAD	coronary artery disease
caspase	cysteine-aspartic proteases
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cells
ESRD	end stage renal disease
FACS	fluorescence-activated cell sorting
FC	fold change
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
HDL	high density lipoprotein
HDL-ACS	HDL isolated from patients with an acute coronary syndrome
HDL-C	high density lipoprotein cholesterol
HDL-CAD	HDL isolated from patients with CAD
HDL-Healthy	HDL isolated from healthy subjects
HDL-sCAD	HDL isolated from patients with stable CAD
HPTR	haptoglobin related protein
ICAM-1	Intercellular Adhesion Molecule 1
ICW	in-cell western
LBP	lipopolysaccharide binding protein
LCAT	lecithin cholesterol acyltransferase

LC-ESI-MS/MS	liquid chromatography–electrospray ionization tandem mass spectrometry
LDL	low density lipoprotein
L-NAME	L-NG-Nitroarginine Methyl Ester
LOX-1	lectin-type oxidized LDL receptor 1
LURIC	Ludwigshafen Risk and Cardiovascular Health
lyso-PC	lysophosphatidylcholine
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MDA	malondialdehyde
MI	myocardial infarction
MPO	myeloperoxidase
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NSTEMI	non-ST segment elevation myocardial infarction
oxLDL	oxidized low density lipoprotein
PAF-AH	platelet-activating factor acetylhydrolase
PI3K	phosphoinositide-3-kinase
PLTP	phospholipid transport protein
PON1	paraoxonase 1
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
PPAR	peroxisome proliferator–activated receptor
PTP	proteotypic peptide
rHDL	reconstituted high density lipoprotein
S1P	sphingosine-1-phosphate
SAA	serum amyloid A
SNP	single nucleotide polymorphism
SR-B	scavenger receptor type B
SR-BI	scavenger receptor type B1
SRM	selected reaction monitoring
STEMI	ST segment elevation myocardial infarction
TLR	toll like receptor
TNF-α	tumor necrosis factor-alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VCAM-1	vascular cell adhesion molecule 1
VLDL	very low density lipoprotein

Contents

Chapter 1

Regulation of endothelial functions by High-density lipoprotein: From physiology to pathophysiology

Meliana Riwanto & Ulf Landmesser

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1.1 Abstract

Epidemiological studies suggest that reduced plasma levels of HDL cholesterol are associated with an increased risk of coronary artery disease and myocardial infarction. Experimental and translational studies have revealed several potential anti-atherogenic effects of HDL, including beneficial effects of HDL on endothelial cell functions. HDL from healthy subjects can exert direct endothelial-atheroprotective effects, such as stimulation of endothelial production of the anti-atherogenic molecule nitric oxide, anti-inflammatory, anti-apoptotic, and anti-thrombotic effects. Furthermore, it has been observed that HDL may stimulate endothelial repair processes, involving mobilisation and promotion of endothelial repair capacity by circulating endothelial repair-promoting mononuclear cells.

However, in recent clinical trials using the HDL cholesterol raising agents torcetrapib, dalcetrapib and niacin, no significant reduction of cardiovascular events was observed in patients with coronary disease. Of note, growing evidence suggests that the vascular effects of HDL can be highly heterogenous and vasoprotective properties of HDL are altered in patients with inflammatory or coronary disease. Characterization of underlying mechanisms and understanding of the clinical relevance of this "HDL dysfunction" are currently an active field of cardiovascular research. A greater understanding of mechanisms of action of HDL and its altered vascular effects is therefore crucial within the context of HDL-targeting therapies. In this chapter, I will address different effects of HDL on endothelial cell functions and how these are altered in patients with cardiovascular disease. Furthermore, mechanisms leading to the heterogeneity of HDL particles and their function will be highlighted.

1.2 Introduction

Epidemiological studies have suggested that reduced plasma levels of HDL cholesterol are associated with an increased risk of coronary artery disease (CAD).[1-6] Moreover, in patients with CAD that are treated intensely with statin and have low levels of low-density lipoprotein (LDL) cholesterol (<70 mg/dL), reduced HDL cholesterol levels were still predictive of major cardiovascular events.[5] In recent years, various biological functions of HDL have been identified, whereby HDL may exert anti-atherogenic effects.[7-9] Besides promoting macrophage cholesterol efflux and reverse cholesterol transport, HDL from healthy subjects has been shown more recently to exert direct vasoprotective effects, such as endothelial-protective, anti-inflammatory and anti-thrombotic effects.[8, 10-13] Accordingly, interventions to increase HDL-cholesterol levels and/or HDL-function are beeing intensely evaluated as a potential therapeutic strategy to reduce cardiovascular risk. However, recent evidence suggests that the vascular effects of HDL are highly heterogenous and vasoprotective properties of HDL may be limited in certain patient populations, such as patients with coronary disease.[14-17]

Several studies have examined the association of genetic variations leading to altered HDL cholesterol plasma levels with coronary disease risk. Polymorphisms in the cholesteryl ester transfer protein (CETP) gene, with a modestly higher HDL cholesterol levels, have been associated with a reduced coronary risk and a lower risk of future myocardial infarction. [18, 19] However, in another study lower plasma levels of HDL cholesterol due to mutations in the ATP binding cassette transporter A1 (ABCA1) gene were not associated with an increased risk of ischemic heart disease.[20] More recently, a mendelian randomization analyses of a relatively common single nucleotide polymorphism in the endothelial lipase gene and other single nucleotide polymorphisms associated with HDL cholesterol suggest that some genetic mechanisms that increase plasma HDL cholesterol levels do not decrease the risk of myocardial infarction.[21]

Recent clinical trials have not been able to demonstrate that some therapies that also increase HDL cholesterol levels reduce cardiovascular risk in patients with coronary disease. The Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) trial testing the impact of the CETP inhibitor torcetrapib on clinical outcome showed an increase in cardiovascular events and total mortality, despite elevations in HDL cholesterol.[22] Dalcetrapib, another CETP inhibitor, raised the HDL-cholesterol level in patients hospitalized with an acute coronary syndrome, but the trial was terminated before completion due to lack of clinical benefit.[23] And very recently, HSP2-THRIVE trial results showed that adding extended-release niacin/laropiprant, another HDL-raising agent, to statins does reduce the risk of cardiovascular disease.[24]

Taken together, these observations likely suggest that plasma HDL cholesterol level alone may not be an optimal therapeutic target. Given the heterogeneity of HDL particles, greater emphasis should therefore

be placed on HDL functionality, especially within the context of HDL-raising therapies, since it is likely, that only raising of HDL with vasoprotective properties can exert cardiovascular protection.

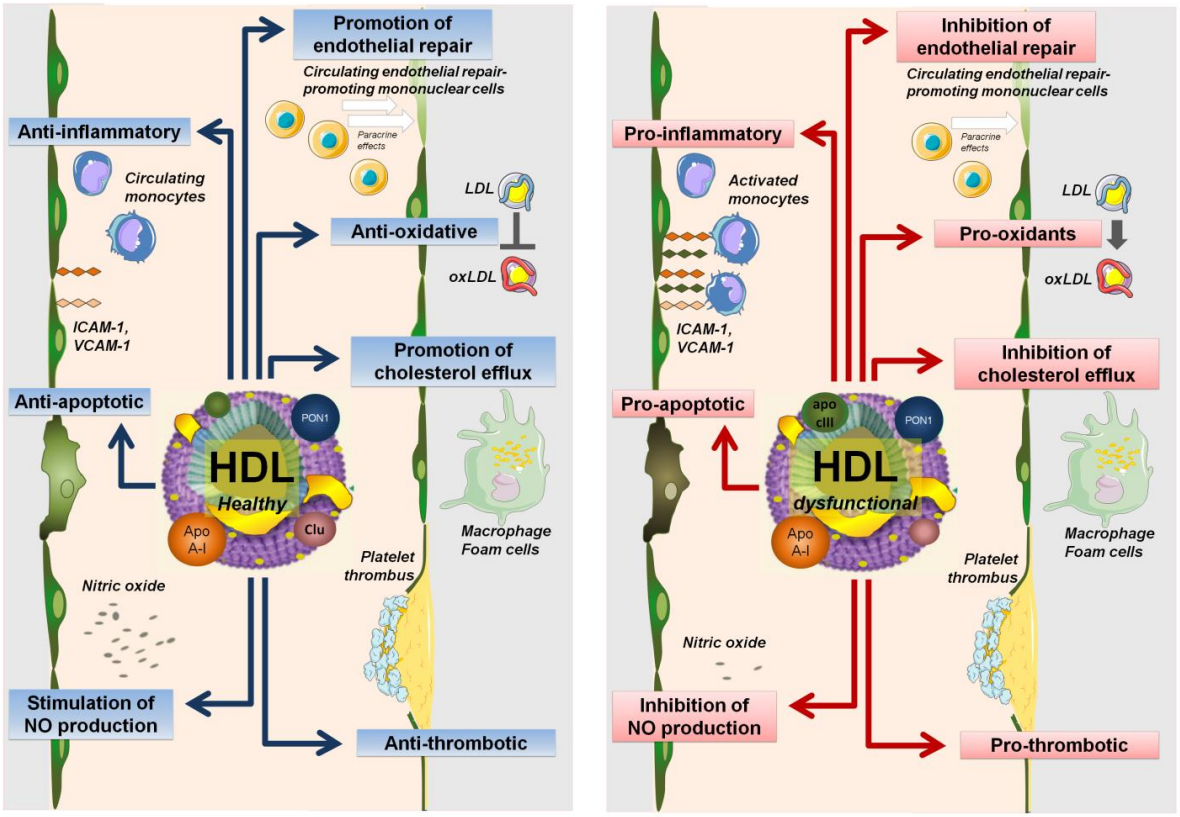

Figure 1.1 *Left panel* – HDL from healthy subjects exerts direct vasoprotective effects, such as endothelial anti-inflammatory, anti-oxidative, anti-apoptotic and anti-thrombotic effects. HDL from healthy subjects stimulates endothelial cell nitric oxide production and promote endothelial repair after vascular injury. *Right panel* - Accumulating evidence suggests that the vascular effects of HDL can be highly heterogeneous. HDL loses important anti-atherosclerotic properties in patients with chronic inflammatory diseases, such as coronary artery disease. These proinflammatory HDL particles have been termed HDL dysfunction.

Importantly, accumulating evidence suggests that the vascular effects of HDL can be highly heterogeneous.[25] We and others have observed that HDL loses important anti-atherosclerotic properties in patients with chronic inflammatory disorders, such as the antiphospholipid syndrome,[26] systemic lupus erythematosus and rheumatoid arthritis,[27] scleroderma,[28] the metabolic syndrome,[29] diabetes,[14, 30] and coronary disease.[16, 17, 31] Notably, in a study of 189 patients with chronic kidney disease on hemodialysis an impaired anti-inflammatory capacity of HDL was correlated with a poor clinical outcome.[32] Furthermore, HDL isolated from subjects with type 1 or type 2 diabetes mellitus or abdominal obesity had reduced capacity to reverse the inhibition of aortic ring endothelium-dependent relaxation by oxLDL as compared to HDL from healthy control subjects.[30, 33, 34] These proinflammatory HDL particles have been termed 'dysfunctional' HDL. The heterogeneity of the vascular effects of HDL may be attributed to

changes in the HDL-associated proteome and lipids, i.e. post-translational protein modifications and changes in the amount and type of proteins and lipids bound to the HDL particle. In particular, high-density lipoprotein is susceptible to oxidation/modification *in vitro* by a variety of oxidants, such as metal ions, peroxyl and hydroxyl radicals, aldehydes, various myeloperoxidase (MPO)-generated oxidants, lipoxygenase, phospholipase A2, elastase, non-enzymatic glycation and homocysteinylation.[35]

In this chapter, I will address different mechanisms whereby HDL exert effects on endothelial cell functions. In particular, the effects of HDL on the regulation of endothelial nitric oxide synthase (eNOS) and endothelial cell NO production, endothelial inflammatory activation, endothelial apoptotic regulation, endothelial repair from vascular injury, lipid oxidation and endothelial thrombotic activation will be discussed. Importantly, recent advances highlighting the pathophysiology of HDL dysfunction affecting these endothelial effects of HDL will be explored.

1.3 Effects of HDL on lipid oxidation

1.3.1 Effects of HDL from healthy subjects on lipid oxidation

Oxidation of LDL has long been suggested as a relevant mechanism for atherogenesis.[36, 37] LDL is entrapped in the subendothelial space where it is subject to oxidative modification through the reactive nitrogen species, myeloperoxidase pathways and others.[38] Once formed, oxidized LDL (oxLDL) is a potent inducer of inflammatory molecules.[39] OxLDL also promotes the differentiation of monocytes into macrophages that take-up oxLDL in a process that converts them into foam cells, hallmark cells of atherosclerotic plaques.[40]

Hessler et al. early on reported that HDL protects against LDL-induced cytotoxicity.[41] HDL was demonstrated to prevent LDL oxidation by cultured endothelial cells.[42-44] HDL is a major carrier of lipid peroxidation products,[45, 46] which are thought to play an important role in the initiation and progression of atherosclerotic vascular disease.[47] HDL can directly inhibit oxidation of low-density lipoprotein via transfer of oxidation products from LDL to HDL.[45] In addition, circulating HDL accumulates oxidized phospholipids, such as hydroperoxides, lysophosphatidylcholine (lyso-PC) and F2-isoprostanes. [45, 46] The transfer of lipid hydroperoxides from LDL prevents the initiation of a free radical chain reaction of oxidation.[48] Furthermore, some of the advanced products of phospholipid oxidation may serve as ligands for scavenger receptor type B (SR-B) and promote uptake of modified lipoproteins by macrophages as well as prothrombotic effects mediated by platelet scavenger receptor CD36.[49, 50]

A study by Navab et al. demonstrated that apoA-I binds to and removes lipid hydroperoxides of LDL in vitro and in vivo.[48] ApoA-I and an apoA-I peptide mimetic removed seeding molecules, hydroperoxyoctadecadienoic acid (HPODE) and hydroperoxyeicosatetraenoic acid (HPETE), from human

LDL, rendering LDL resistant to oxidation by human artery wall cells.[48] Furthermore, Injection of apoA-I, but not apoAII or murine serum albumin, into mice rendered their LDL resistant to cell-mediated oxidation. Infusion of apoA-I into humans also rendered their LDL resistant to cell-mediated oxidation.[48] Treatment of human artery wall cells with apoA-I, but not apoA-II, or treatment with an apoA-I peptide mimetic, or with normal HDL, or paraoxonase, also rendered the cells unable to oxidize LDL.[51]

Human HDL can also directly reduce cholesteryl ester hydroperoxides and phosphatidylcholine hydroperoxides via Met residues 112 and 148 of apoA-I.[52] Recombinant HDL containing only apoA-I and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) was as effective as native HDL in preventing LDL oxidation, supporting a key anti-oxidant role for apoA-I.[53] Multiple *in vivo* studies have demonstrated that apoA-I is a potent anti-oxidative, anti-inflammatory and anti-atherosclerotic agent.[7, 54-58] Recent clinical studies have demonstrated that although very high HDL–cholesterol and large-size HDL particles are associated with a twofold increase in cardiovascular risk,[59] a high concentration of apoA-I is an independent negative predictor of cardiovascular risk.[59, 60]

Interestingly, several other HDL-associated apolipoproteins have also been shown to exert anti-oxidant effects. ApoA-II-enriched HDL from mice transgenic for human apoA-II protected VLDL from oxidation more efficiently than control HDL.[61] However, in other studies, overexpression of human apoA-II in dyslipidemic mice accelerates atherosclerosis and reduces antioxidative activity of HDL.[62, 63] The authors postulated that the proatherogenic actions of apoA-II may be related to the displacement of apoA-I and PON1 by apoA-II from HDL particles.[62]

Meanwhile, apolipoprotein E has been shown to have allele-specific anti-oxidant activity.[64] Apolipoprotein E2 stimulates endothelial nitric oxide (NO) release and has anti-inflammatory activities.[65] In contrast, apolipoprotein E4 has been described as pro-inflammatory.[66] It has also been reported that HDL-associated apolipoprotein J can inhibit LDL oxidation by artery wall cells.[67] In addition, apoA-IV has been demonstrated to exert anti-oxidant, anti-inflammatory and anti-atherosclerotic actions *in vivo.*[68-70]

Notably, HDL carries other anti-oxidant enzymes that may be involved in prevention of lipid oxidation or degradation of lipid hydroperoxides, such as PON1, LCAT and platelet-activating factor acetylhydrolase (PAF-AH). In particular, PON-1 has been suggested to be an important regulator of the anti-atherogenic capacity of HDL.[71, 72] Various studies have suggested that the direct anti-oxidant effect of HDL on LDL oxidation, measured as a reduction in lipid peroxides, is to a significant extent mediated by PON1.[73-75] PON1 enhances cholesterol efflux from macrophages by promoting HDL binding mediated by ABCA1.[76] This effect of PON1 can indirectly reduce pro-inflammatory signalling in cells *in vivo* and contribute to the anti-atherosclerotic effects of PON1.[71, 72] Mice deficient in PON1 displayed significantly larger aortic atherosclerotic lesions as compared to their wild type controls and HDL isolated from PON1-deficient

mice was unable to prevent LDL oxidation in a cell co-culture model of the arterial wall.[71] Moreover, signs of oxidative stress, vascular inflammation and thrombotic activation have been observed in PON1-deficient mice.[77] In human studies, higher PON1 activity is associated with a lower incidence of major cardiovascular events.[78] Pathological conditions associated with oxidative stress, such as chronic renal failure, rheumatoid arthritis and Alzheimer's disease, are frequently associated with reduced activity of PON1.[78] Furthermore, a pro-atherosclerotic high-fat diet leads to reduced PON1 activity.[78]

The anti-oxidant role of LCAT has been nicely illustrated by a study demonstrating its capacity to directly hydrolyse oxidized polar phospholipids.[79] PAF-AH is another HDL-associated enzyme that can hydrolyse oxidized phospholipids.[80, 81] Lipoproteins isolated from mice expressing human PAF-AH are more resistant to oxidative stress, inhibit foam cell formation and promote cholesterol efflux in macrophages.[81] In arteries of non-hyperlipidaemic rabbits, local expression of PAF-AH reduced the accumulation of oxidatively modified LDL without changing plasma levels of PAF-AH and reduced the expression of endothelial cell adhesion molecules.[82] In human studies, PAF-AH deficiency through a missense mutation of the gene is an independent risk factor for coronary artery disease in Japanese men.[83] Circulating levels of PAF-AH is also shown to be an independent marker of the risk of CAD.[84]

HDL has also been shown to promote efflux of 7-ketocholesterol[85, 86] at sites of inflammation and thereby reduces endothelial cell inflammatory activation.[87] Accordingly, Nicholls et al. have reported that reconstituted HDL inhibits superoxide production and vascular inflammation induced by a non-occlusive carotid periarterial collar in normocholesterolemic rabbits.[88] In addition, Van Linthout et al. have observed that human apoA-I gene transfer in rats with streptozotocin-induced diabetes mellitus resulted in a 1.9-fold increase in HDL cholesterol levels and inhibition of angiotensin II type 1 receptor-mediated NAD(P)H oxidase activation and generation of reactive oxygen species.[89]

1.3.2 Alterations of the effects of HDL on lipid oxidation after surgery and in CAD or diabetes

Early studies by van Lenten et al. have demonstrated that the anti-inflammatory capacity of HDL is affected by acute phase responses in both humans and rabbits.[90] The authors isolated human HDL from the patients before and immediately after surgery and characterized the effects of HDL on LDL-induced monocyte transmigration and lipid hydroperoxide formation.[90] Before cardiac surgery, HDL completely inhibited the LDL-induced increase in monocyte transmigration and lipid hydroperoxide formation. In marked contrast, "acute phase" HDL obtained from the same patients 2-3 days after surgery amplified the LDL-induced monocyte transmigration and was less effective in inhibiting lipid hydroperoxide formation, i.e. HDL in the same patient had been transformed from an anti-inflammatory towards a pro-inflammatory particle.[90] Interestingly, the changes in HDL functionality in this study were paralled by an increase in

HDL-associated acute phase reactants (i.e. coeruloplasmin and serum amyloid A), while the activities of the HDL-associated anti-oxidant enzymes paraoxonase and platelet-factor activating acetylhydrolase were reduced in acute phase HDL.[90] Similarly, an acute Influenza A infection in wild type mice progressively impaired the ability of HDL to inhibit LDL oxidation and LDL-induced monocyte chemotactic activity in human artery wall cell cocultures up to 9 days after inoculation.[91]

In marked contrast to the anti-oxidative and anti-inflammatory effects of HDL from healthy subjects, HDL from normolipidemic patients with angiographically confirmed CAD increased LDL-induced monocyte chemotactic activity by human artery wall cells and promoted LDL oxidation.[92] A subsequent study by Ansell et al suggested that the capacity of HDL to alter LDL-induced monocyte chemotactic activity in patients with CAD was somewhat improved after 6 weeks of simvastatin therapy.[31] However, HDL from patients with CAD on statin therapy remained proinflammatory in contrast to HDL from age- and sex-matched healthy subjects.

Navab et al. developed a fluorescent cell-free assay to detect the capacity of HDL to inhibit the oxidation of LDL, or inhibit the oxidation of l-α-1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC) by hydroperoxyoctadecadienoic acid (HPODE), or inactivate oxidized PAPC (Ox-PAPC).[93] Using this assay, HDL isolated from 27 patients with coronary atherosclerosis failed to inhibit the fluorescent signal generated by a control LDL, whereas HDL from 31 matched normal subjects with the same levels of HDL cholesterol significantly inhibited the signal.[93]

The presence of oxidized lipids in HDL has been proposed to play a role in the altered anti-oxidant properties of HDL.[94] Administration of apoA-1 mimetic peptides, L-4F, to apoE deficient mice has been shown to reduce plasma levels of oxidized fatty acids (15-HETE, 5-HETE, 13-HODE and 9-HODE) and improve the HDL anti-oxidant capacity and the capacity of HDL to inhibit LDL-induced monocyte chemotactic activity in cultured human aortic endothelial cells.[95] Moreover, in a recent study by Morgantini et al., HDL from patients with type 2 diabetes had impaired anti-oxidant properties and increased oxidized fatty acids content.[96] The authors postulated that elevated content of oxidized fatty acids (5-HETE, 9-HETE, 12-HETE, 15-HETE, 9-HODE, and 13-HODE) in HDL isolated from the type 2 diabetics patients may account for the impaired anti-oxidant properties of the lipoprotein.[96]

1.4 Effects of HDL on endothelial NO-synthase dependent Nitric Oxide production and alterations in cardiovascular disease

1.4.1 Effects of HDL from healthy subjects on endothelial NO synthase

Endothelial nitric oxide (NO) plays a crucial role in the regulation of vascular tone and structure. Endothelial NO synthase derived NO has been shown to exert a variety of atheroprotective effects in the

vasculature, such as anti-inflammatory and anti-thrombotic effects.[97] Reduced endothelial NO bioavailability has therefore been suggested to promote initiation and progression of atherosclerosis.[97]

Accumulating evidence suggests that HDL can directly stimulate endothelial NO synthase mediated NO production as well as induce endothelium dependent, NO-mediated vasodilation via endothelial SR-BI.[10] Of note, experimental studies have consistently demonstrated the capacity of HDL to modulate eNOS expression and to stimulate endothelial NO production *in vitro* and *in vivo*.[11, 14, 16, 98-100] Moreover, in human studies, administration of reconstituted HDL has been shown to improve endothelial function in subjects with hypercholesterolemia and in subjects with isolated low HDL due to heterozygous loss-of-function mutations in the ABCA-1 gene locus.[101, 102]

Several mechanisms have been proposed to account for the endothelial NO-stimulating capacity of HDL. Early studies have shown that HDL prevents oxidized LDL-mediated eNOS displacements from caveolae and restores enzyme stimulation.[103] A study by Yuhanna et al. suggested that HDL can bind to endothelial SR-BI and thus directly stimulate eNOS-mediated NO production.[10] HDL binding to SR-BI initiates tyrosine kinase Src-mediated activation of phosphoinositide (PI) 3-kinase, which in turn activates Akt and the MAP kinase/extracellular signal-regulated kinase pathway.[99] Activation of endothelial Akt by HDL stimulates phosphorylation of eNOS at serine residue 1177,[11, 99] which is known to be an important regulatory mechanism leading to eNOS activation.[104]

Another mechanism has also been identified whereby HDL can maintain endothelial cell NO production and availability in mice fed a high-cholesterol diet.[85] These authors suggested that HDL-induced ABCG1-mediated efflux of oxysterols from endothelial cells plays a role since 7-ketosterol, a dietary oxysterol, accumulated in endothelial cells of ABCG1- deficient mice on a western diet.[85] Interestingly, incubation of human aortic endothelial cells with HDL prevented 7-ketosterol-induced production of reactive oxygen species and disruption of the active eNOS dimer. Furthermore, HDL-mediated cholesterol efflux via ABCG-1 reduced the inhibitory interaction of eNOS with caveolin-1 and thereby restored eNOS activity in cholesterol-loaded endothelial cells.[105] These data suggest that the ability of HDL to preserve endothelial function in the presence of hypercholesterolemia may, at least in part, relate to an increased endothelial efflux of oxysterols.

Various components of HDL have been suggested to play a role in its endothelial NO-stimulating capacity. In cultured endothelial cells, the potential interaction of apoA-I with eNOS has been reported.[100] However, despite being the major SR-BI ligand of HDL, lipid-free apoA-I failed to activate eNOS, suggesting that other HDL components are likely important for the eNOS-stimulating capacity of HDL. In isolated endothelial cell plasma membranes, anti-apoA-I antibody inhibits eNOS activation by HDL, whereas anti-apoA-II antibody further enhances eNOS stimulation by HDL.[10] Several studies have

suggested that HDL-associated lysophospholipids may play a role in eNOS activation. Of note, HDL-associated sphingosylphosphorylcholine, sphingosine-1-phosphate, lysosulfatide may cause eNOS-dependent relaxation of precontracted aortic rings from mice via binding to the lysophospholipid receptor S1P3 expressed on endothelial cells.[11] The vasodilatory response to HDL, however, was not completely inhibited in S1P3 deficient mice.[11]

Recently, we observed that the HDL-associated antioxidant enzyme paraoxonase (PON) 1 as an important determinant of the capacity of HDL to stimulate endothelial NO production and to exert NO-dependent endothelial- atheroprotective effects.[16] Inhibition of PON1 in HDL from healthy subjects impaired the capacity of HDL to stimulate endothelial NO production and HDL isolated from PON1-deficient mice failed to stimulate NO production in mouse aortic endothelial cells.[16] Furthermore, inhibition of eNOS-mediated NO production prevented the inhibitory effects of HDL from healthy subjects on nuclear factor kB (NF-kB) activity, vascular cell adhesion molecule (VCAM)-1 expression and endothelial monocyte adhesion, suggesting that the capacity of HDL to stimulate endothelial NO production is important for these endothelial anti-inflammatory effects of HDL.[16]

1.4.2 Alterations of the effects of HDL on eNOS in patients with cardiovascular disease

We and others have recently shown that direct endothelial effects of HDL from patients with CAD, diabetes, or antiphospholipid syndrome are markedly altered when compared to HDL from healthy subjects. In contrast to HDL from healthy subjects, HDL from patients with diabetes and low levels of HDL cholesterol failed to stimulate endothelial cell NO production and to promote endothelial repair in a carotid artery injury model in mice.[14] We also reported that HDL from patients with antiphospholipid syndrome had reduced nitric oxide bioavailability and had impaired anti-inflammatory and antioxidant properties. Moreover, HDL from patients with either stable CAD or an acute coronary syndrome, in contrast to HDL from age- and gender-matched healthy subjects, inhibited rather than stimulated endothelial cell NO production and lost the capacity to limit endothelial inflammatory activation as well as to promote endothelial repair in vivo.[16]

Notably, we have observed that malondialdehyde (MDA) content is elevated in HDL from coronary disease patients compared with HDL from healthy subjects, which blunts endothelial NO production.[16] The antagonistic action of MDA was determined to be mediated by lectin-type oxidized LDL receptor 1 activation of protein kinase C-βII, which inhibits Akt-activating phosphorylation (Akt- Ser473) and eNOS-activating phosphorylation at Ser1177. Because MDA formation is decreased by HDL-associated paraoxonase 1 (PON1),[106] PON1 activity was evaluated and was found to be markedly decreased in HDL from patients with coronary disease.[16] Furthermore, PON1 inactivation in HDL from healthy subjects results in greater protein kinase C-βII activation in cultured endothelial cells, decreased activating eNOS-

Ser1177 phosphorylation, and increased inactivating eNOS-Thr495 phosphorylation, resulting in attenuated NO production. Furthermore, HDL from PON1-deficient mice failed to stimulate endothelial cell NO production.[16] These observations suggest that alterations of HDL-associated PON1 may have a major impact on endothelial effects of HDL.

An inverse relationship between PON1 serum activity and cardiovascular events has previously been reported.[107, 108] A recent analysis of SNPs for paraoxonase-1 identified in genome wide association studies did not reveal a significant association between the lead SNPs for paraoxonase-1, that was associated with mildly reduced paraoxonase activity, and the risk of cardiovascular events.[109] A difficulty with respect to paraoxonase-1 is that it is not known to what extent the paraoxon and arylesterase activity of the enzyme represent biologically relevant functions. We and others have observed important post-translational modifications of the enzyme, that could lead to further alterations of biological properties of the enzyme.[16, 110]

1.5 Effects of HDL on endothelial inflammatory activation

1.5.1 Effects of HDL from healthy subjects on endothelial inflammatory activation

Atheroscleorsis is a chronic inflammatory disease. Endothelial adhesion and subsequent infiltration and accumulation of monocytes/macrophages and T lymphocytes into the arterial intima represent critical steps in initiation and progression of atherosclerotic lesions.[47] HDL has been shown to inhibit the expression of monocyte chemoattractant protein (MCP)-1, an important pro-inflammatory chemokine in endothelial cells.[94, 111] Studies have demonstrated that native HDL and reconstituted HDL containing apoA-I or the apoA-I Milano mutant inhibit the expression of leukocyte adhesion molecules in endothelial cells that are activated by pro-inflammatory stimuli.[112-115] Furthermore, HDL has been suggested to inhibit endothelial monocyte adhesion induced by oxidized LDL[116] or TNF-α[115] and monocyte transmigration in co-cultures of human aortic endothelial cells and smooth muscle cells stimulated with LDL.[94]

The anti-inflammatory effects of HDL have also been demonstrated by several *in vivo* studies. Administration of reconstituted human HDL in apoE-deficient mice reduced VCAM-1 expression and decreased monocyte/macrophage infiltration following carotid artery cuff injury.[117] Recently, it was shown that apoA-I gene transfer caused not only an increased HDL cholesterol plasma levels, but also inhibited diabetes-induced myocardial mRNA expression of VCAM-1 and ICAM-1 in mice with streptozotocin-induced diabetic cardiomyopathy.[118] In contrast, in apoE-deficient mice with transgenic overexpression of human apoA-I, endothelial VCAM-1 expression was not reduced in the aortic branch sites and was not associated with reduced monocyte adherence, despite reducing aortic atherosclerotic lesion formation.[119] Hence, the anti-inflammatory capacity of HDL may be heterogeneous. This is consistent with findings of recent studies demonstrating that the inhibitory effects of HDL isolated from different human subjects on

TNF-α stimulated endothelial VCAM-1 expression varied considerably.[90, 120] In human studies, it has been suggested that administration of reconstituted HDL increased the anti-inflammatory capacity of HDL from patients with type-2 diabetes.[121]

Several mechanisms have been suggested to explain the inhibitory effects of HDL on endothelial inflammatory activation.[9] HDL can inhibit activation of the endothelial pro-inflammatory transcription factor NF-κB.[114, 115] Interestingly, impaired endothelial NO bioavailability and increased endothelial superoxide production have been implicated in activation of NF-κB.[122] Furthermore, it was demonstrated that endothelial anti-inflammatory effects of HDL are mediated via SR-BI, PDZK1, PI3 kinase, eNOS, and S1P receptors.[123] Recently, we have observed that inhibition of eNOS-mediated NO production prevented the inhibitory effects of HDL from healthy subjects on nuclear factor kB (NF-kB) activity, vascular cell adhesion molecule (VCAM)-1 expression and endothelial monocyte adhesion, suggesting that the capacity of HDL to stimulate endothelial NO production is important for these endothelial anti-inflammatory effects of HDL.[16]

It has been proposed that apoA-1, the major protein constituent of HDL, is able to recapitulate the anti-inflammatory capacity of HDL. In an *in vivo* study, infusion of apoA-I to rabbits subjected to acute vascular inflammation reduced neutrophil infiltration and endothelial cell inflammatory activation.[124] Furthermore, administration of apoA-1 mimetic peptides, D-4F and L-4F has been shown to reduce vascular inflammation in type I diabetic rats and improved insulin sensitivity in obese mice.[125, 126] Furthermore, lipid-free apoA-I and rHDL treatment reduced the expression of chemokines and chemokines receptors *in vivo* and *in vitro* via modulation of NF-κB and peroxisome proliferator–activated receptor γ.[127] One of the potential mechanisms for the anti-inflammatory effect of apoA-I is by mediating cellular cholesterol efflux through ABCA1, an ATP-binding transporter.[128, 129] Interestingly, apoA-I has also been shown to attenuate palmitate-induced NF-kB activation by reducing toll-like receptor-4 recruitment into lipid rafts.[130]

Besides apoA-I, the lipid component of HDL has also been proposed to be important for the anti-inflammatory effects of HDL. *In vitro* studies using discoidal reconstituted HDL containing apoA-I as the sole protein suggested that inhibitory effects of HDL on endothelial cell adhesion molecule expression are also, at least in part, dependent on HDL-associated phospholipid species.[131] The inhibition of cytokine induced expression of VCAM-1 by reconstituted HDL varied substantially when different phosphatidylcholine species were compared, indicating that the lipid composition of HDL influences its anti-inflammatory capacity and might be an important determinant of HDL functionality.[9, 131]

1.5.2 Alterations of endothelial anti-inflammatory effects of HDL

Early studies have demonstrated that the anti-inflammatory capacity of HDL is lost during acute phase responses in both humans and rabbits.[90, 91] Furthermore, the capacity of HDL to inhibit LDL-induced monocyte chemotactic activity has also been shown to be impaired in CAD patients,[92] which can be improved following simvastatin therapy.[31] Recent studies have also described the loss of HDL anti-inflammatory capacity in patients with CAD, diabetes, as well as end-stage renal disease.[16, 96, 132]

Various mechanisms have been proposed to account for the impaired endothelial anti-inflammatory effects of HDL. The decrease in HDL apoA-I levels in inflammatory states has been related to decreased apoA-I synthesis in the liver, accelerated HDL catabolism, and apoA-I replacement in HDL particles by serum amyloid A (SAA).[133, 134] Upon induction of the acute phase, SAA is able to replace apoA-I in small, dense HDL, resulting in reduced plasma levels of apoA-I.[135-137] In rabbits and mice, SAA can completely replace apoA-I in a subset of small, dense HDL particles, therefore functioning as a structural apolipoprotein.[137, 138] Accordingly, SAA was recently found to be enriched in HDL from ESRD patients, which correlated with its reduced anti-inflammatory capacity.[132]

In addition, certain amino acid residues in apoA-I, such as methionine, cysteine, tyrosine, and lysine residues are susceptible to oxidative modifications[139-141] ApoA-I isolated from plasma and human atherosclerotic lesions contain oxidized amino acid residues, including chlorotyrosines, nitrotyrosines and oxidized lysine and methionine residues.[139, 140, 142] *In vitro* studies have demonstrated that MPO-catalysed oxidative modification of HDL or apoA-I leads to the loss of ABCA1-dependent cholesterol efflux function of the lipoprotein and converts HDL into a pro-inflammatory particle which promotes NF-κB activation and endothelial VCAM-1 expression [139, 140, 143]

Glycation of HDL and apoA-I, a process that is known to occur in diabetes *in vivo*,[144] has also been proposed to impact on the anti-inflammatory capacity of HDL.[145] In contrast to normal lipid-free Apo-AI, glycated lipid-free Apo-AI infusion did not decrease adhesion molecule expression following vascular injury.[145] Glycation of HDL has also been shown to impair the HDL capacity to inhibit oxLDL-induced monocyte adhesion to human aortic endothelial cells in vitro.[146]

1.6 Effects of HDL on endothelial apoptotic regulation

1.6.1 Effects of HDL from healthy subjects on endothelial apoptosis

Endothelial dysfunction and injury contribute importantly to the pathogenesis of atherosclerosis and other vascular disorders.[97, 147, 148] Experimental studies have shown that atherosclerotic lesion-prone vascular regions are characterized by a high endothelial cell turn-over,[149] which has been attributed to an increased rate of endothelial cell apoptosis. Endothelial cell apoptosis has therefore been suggested to contribute

importantly to the pathophysiology of coronary disease.[150-153] The capacity of HDL to attenuate endothelial cell apoptosis may therefore represent a potentially important anti-atherogenic property of HDL.[154-157]

HDL inhibits apoptosis of endothelial cells induced by both death-receptor-mediated and mitochondrial mediated apoptotic pathways. HDL may inhibit apoptosis triggered by various proatherogenic factors, such as TNF-α, oxidized LDL and growth factor deprivation.[154-156] Both HDL-associated proteins and lipids have been suggested to contribute to the anti-apoptotic capacity of HDL. ApoA-I has been shown to inhibit of endothelial cell apoptosis induced by oxidized LDL, VLDL, and TNF-α.[155, 156, 158] HDL subpopulations enriched with apoA-I account for approximately 70% of the anti-apoptotic activity of HDL in human microvascular endothelial cells that were treated with mildly oxidized LDL and reconstitution of HDL with apoA-I, cholesterol and phospholipids potently decreased oxidized LDL-induced apoptosis in these cells,[157] suggesting that apoA-I plays an important role for the anti-apoptotic capacity of HDL in oxidized LDL-stimulated endothelial cells. We have recently identified HDL-associated clusterin and apolipoprotein C-III to be implicated in the regulation of endothelial apoptosis in CAD.[17] Our results suggest that reduced clusterin and increased apolipoprotein C-III content in HDL isolated from patients with CAD alters the effects of HDL on endothelial anti- and pro-apoptotic signaling pathways, leading to a loss of the endothelial anti-apoptotic capacity of HDL in patients with CAD.[17]

HDL-associated lysosphingolipids have also been shown to inhibit endothelial cell apoptosis triggered by growth factor deprivation[154, 159, 160] The anti-apoptotic capacity of HDL-associated lipids was further supported by the findings that the ratio of sphingosine-1-phosphate and sphingomyelin was increased in small dense HDL3 particles and positively correlated with the capacity of these HDL subpopulations to attenuate endothelial cell apoptosis.[161]

Several mechanisms have been proposed for the endothelial anti-apoptotic effects of HDL, depending on the trigger of apoptosis. OxLDL causes a delayed but sustained increase in intracellular calcium in endothelial cells, leading to cell death, and this effect is reversed by HDL and mediated by prevention of the calcium increase.[156] Tumor necrosis factor-α–induced endothelial cell apoptosis is also inhibited by HDL, and this is associated with attenuated induction of CPP32-like protease (caspase 3), which is a component of all primary apoptotic pathways.[155] Growth factor deprivation activates the mitochondrial pathway of apoptosis, which can be suppressed by HDL. HDL inhibits the dissipation of mitochondrial potential, oxygen-derived free radical generation, cytochrome c release to the cytoplasm, and activation of caspase 3 and caspase 9. HDL also activates Akt and causes phosphorylation of the Akt target Bcl-2-associated death promoter Bad, preventing it from binding to Bcl-xL. Bcl-xL, an anti-apoptotic Bcl-2 family protein, is then free to inhibit mitochondria-mediated apoptosis.[154] In addition, HDL causes phosphoinositide 3 (PI3) kinase mediated up-regulation of the Bcl-xL expression.[17] Interestingly, HDL

retained its anti-apoptotic activity after knockdown of eNOS using specific RNA interference or pharmacological inhibition using L-NAME,[17] suggesting that HDL may exert its anti-apoptotic activity independently of eNOS activation. The lysophospholipid sphingosine-1-phosphate (S1P) enhances endothelial cell survival, and these effects are inhibited by knockdown of the S1P receptor endothelial differentiation gene-1/S1P1 by pertussis toxin and by phosphoinositide 3 (PI3) kinase and Erk pathway antagonists, suggesting that signaling by lysophospholipid components of HDL may be important for the inhibition of apoptosis.[160]

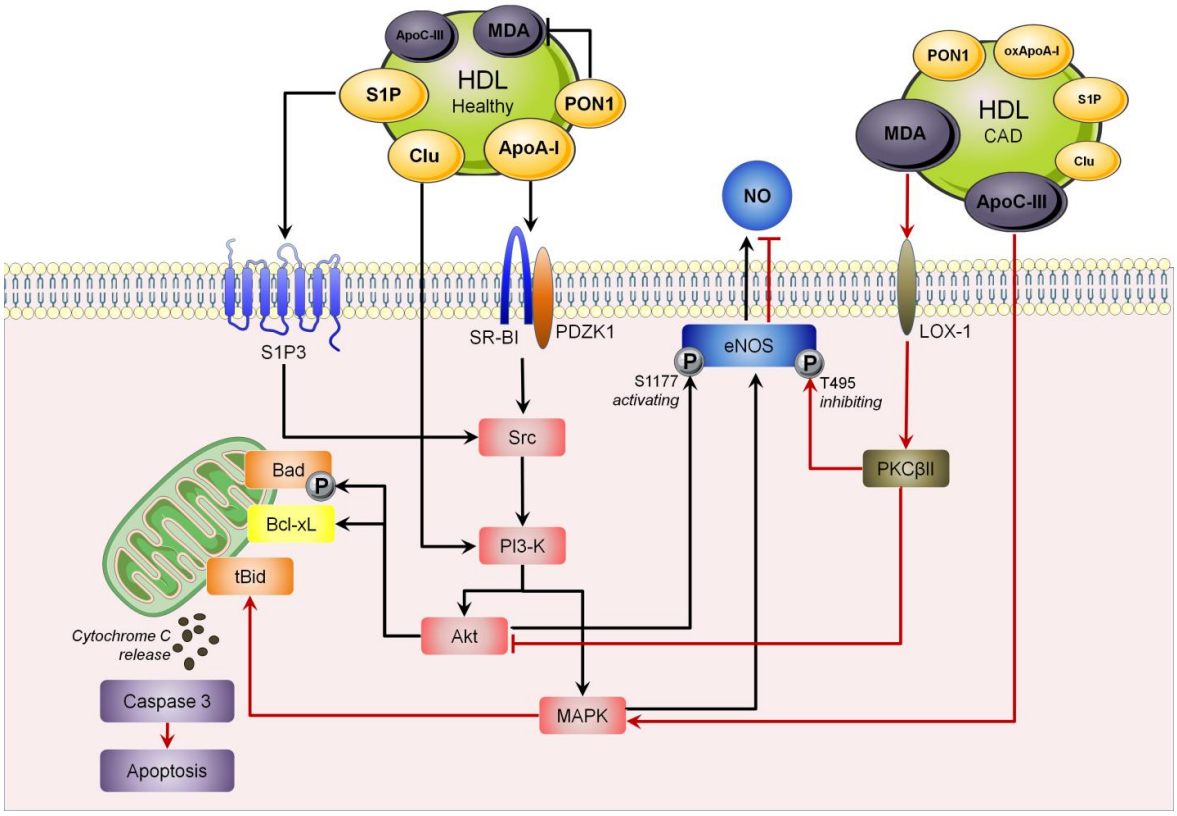

Figure 1.2 Signaling pathways mediating the effects of HDL on endothelial NO production and endothelial apoptosis. HDL from healthy subjects binds to SR-BI via apoA-I, leading to PDZK1-dependent activation of Src family kinases, PI3K and Akt, which phosphorylates eNOS at serine residue 1177, therefore increasing eNOS activity. PI3K-dependent MAPK activation and binding of HDL-associated lysophospholipids to the S1P3 receptor also activate eNOS. In contrast, HDL from patients with CAD suppresses eNOS activation. Inactivation of PON1 and greater accumulation of MDA in HDL lead to LOX-1-mediated activation of PKCβII and inhibit phosphorylation of eNOS at threonine 495. HDL-associated clusterin promotes endothelial anti-apoptotic signaling via activation of PI3K and Akt leading to increased expression of anti-apoptotic Bcl-xL. Akt activation also phosphorylates Bcl-2-associated death promoter Bad, preventing it from binding to Bcl-xL, which is then free to inhibit mitochondria-mediated apoptosis. In CAD, the level of HDL-associated clusterin is reduced, whereas HDL-associated apoC-III content is increased. HDL-associated apoC-III activates MAPK signaling via phosphorylation of p38 leading to increased activation of pro-apoptotic tBid, which promotes cytochrome C release from the mitochondria and downstream caspase-3 mediated apoptosis.

1.6.2 Alterations of the effects of HDL on endothelial apoptosis in cardiovascular disease

Recently, we have observed that reduced clusterin and increased apolipoprotein C-III content in HDL isolated from patients with CAD lead to activation of pro-apoptotic signaling pathways in endothelial cells.[17] In contrast to HDL from healthy subjects, HDL isolated from patients with stable CAD or an acute coronary syndrome failed to inhibit endothelial cell apoptosis *in vitro* and in apoE-deficient-mice *in vivo*. Instead, HDL isolated from these patients stimulated endothelial pro-apoptotic pathways, in particular p38-MAPK-mediated activation of the pro-apoptotic Bcl-2-protein tBid. Our studies further suggest that differences in the proteome of HDL from patients with CAD, in particular reduced HDL-associated clusterin and increased HDL-associated apoC-III, play an important role for altered activation of endothelial anti- and pro-apoptotic signaling pathways.[17] Furthermore, oxidative modifications of HDL may also play a role in the loss of anti-apoptotic activity of HDL, as demonstrated by Undurti et al. showing that MPO-catalyzed oxidation of HDL resulted in the impaired capacity to inhibit endothelial apoptosis *in vitro*.[143]

1.7 Effects of HDL on endothelial repair after vascular injury

1.7.1 Effects of HDL from healthy subjects on endothelial repair processes

Recent studies have also suggested that HDL may stimulate endothelial repair processes. Endothelial repair process have long been thought of to be only dependent on the proliferation and migration of local adjacent endothelial cells,[149] however several recent studies have clearly demonstrated that bone-marrow derived endothelial progenitor cells (EPC) may promote endothelial repair after vascular injury,[162-164] contribute to endothelial repair processes in lesion-prone areas of experimental atherosclerosis and improve endothelial function.[165, 166]

HDL stimulates endothelial repair by promotion of endothelial cell proliferation or migration and stimulation of the recruitment and endothelial repair capacity of EPC.[14, 167, 168] HDL induces a marked increase in endothelial cell migration *in vitro* with effects comparable to endothelial growth factors, such as basic fibroblast growth factor or vascular endothelial growth factor.[160, 167, 169] Native HDL and the HDL-associated lysosphingolipid sphingosine-1-phosphate stimulate endothelial cell migration via sphingosine-1-phosphate receptors S1P1 and S1P3 and the effects could be blocked by pertussis toxin which inhibits the interactions between G proteins and G protein-coupled receptors.[160] The importance of sphingosine-1-phosphate for endothelial cell migration was supported by another study demonstrating that sphingosine-1-phosphate induced tube formation of human coronary artery endothelial cells *in vitro* by Ras/Raf1-dependent ERK activation.[170] In contrast, in a work by Seetharam et al. pertussis toxin did not affect HDL-mediated endothelial cell migration,[167] suggesting the presence of another pathway and agonist, which induces the migration of endothelial cells by HDL. Indeed, the authors observed that reconstituted HDL

consisting of apoA-I, palmitoyloleylphosphatidylcholine and cholesterol was able to induce endothelial cell migration[167] Moreover, native HDL induced rapid changes in the actin cytoskeleton of endothelial cells (i.e. a decrease in stress fibers, an increase in lamellipodia, and membrane ruffling) paralleled by an activation of the small GTPase Rac, that is known to mediate lamellipodia formation.[167] Interestingly, the authors were able to demonstrate that endothelial Rac activation and migration in response to HDL is independent of endothelial NO production, but requires binding of HDL to SR-BI and activation of Src kinase, PI3-kinase and MAP kinase.[167] PDZ domain-containing protein PDZK1 was identified as an adaptor protein of SR-BI in endothelial cells and it was suggested that PDZK1 is required for the initiation of HDL signalling *via* SR-BI in endothelial cells and plays an important role for endothelial cell migration induced by HDL.[168] Interestingly, further studies have suggested that HDL and SR-BI promote re-endothelialization of the carotid artery after perivascular electric injury in mice.[14, 167] In this model, carotid artery re-endothelialization was impaired in apoA-I deficient mice with low HDL levels as well as in SR-BI deficient mice. Of note, reconstitution of apoA-I expression by liver-directed apoA-I gene transfer with subsequent normalisation of HDL plasma levels restored the re-endothelialization response in apoA-I deficient mice, strongly suggesting that apoA-I and HDL promote endothelial monolayer integrity *in vivo.*[167] In another study, elevation of HDL levels in apoE-deficient mice induced by adenoviral human apoA-I (AdA-I) transfer increased the number of Flk1 / Sca-1 double-positive cells in peripheral blood and the number of DiIacLDL / FITC-isolectin double positive cells after 4 days of *ex vivo* culture of spleen mononuclear cells.[171]

Besides increasing the number of EPC, AdA-I transfer in apoE-deficient mice improved the migratory capacity of spleen-derived early EPC in response to HDL, the adhesion of spleen-derived early EPC to fibronectin and the invasion of spleen-derived early EPC in solidified Matrigel.[171] Finally, AdA-I transfer also promoted the incorporation of EPCs in Balb/c common carotid artery allografts transplanted paratopically in C57BL/6 ApoE-/- mice that was associated with an increase in endothelial regeneration and inhibition of transplant arteriosclerosis.[171] In a follow-up study, the same group observed that murine and human early EPC express SR-BI, as indicated by immunocytochemistry analysis of human early EPCs and murine spleen-derived early EPCs after 4 and 7 days of *ex vivo* culture.[172] Of note, the authors did not observe an increase in circulating Flk1 / Sca-1 double-positive cells and DiI-acLDL / FITC-isolectin double positive cells after *ex vivo* culture of spleen mononuclear cells after AdA-I transfer in mice transplanted with SR-BI deficient bone marrow, suggesting that expression of SR-BI in bone marrow is critical for EPC mobilisation induced by HDL.[172] Furthermore, the migratory capacity of bone-marrow derived early EPC deficient in SR-BI in response to HDL was reduced as compared to early EPC containing SR-BI and this was at least in part due to an impaired activation of extracellular signal-regulated kinases (ERK) and decreased NO production in SR-BI deficient early EPC.[172] *In vivo*, SR-BI deficiency in bone marrow abrogated the inhibitory effect of AdA-I transfer on allograft vasculopathy

after paratopical transplantation of a common carotid artery of a female BALB/c donor mouse into the recipient male C57BL/6 mice, that was paralleled by impaired endothelial regeneration and EPC incorporation in allografts.[172] Besides increasing HDL levels by adenoviral human apoA-I transfer, intravenous infusion of reconstituted HDL has also been shown to increase the number of Sca-1 positive cells in the aortic endothelium of apoE deficient mice, supporting a role for HDL in promoting progenitor-mediated endothelial repair.[173] Furthermore, intravenous injection of reconstituted HDL increased blood flow recovery and capillary density in a murine ischemic hindlimb model.[174]

Of note, a pivotal role for eNOS in the regulation of EPC mobilization was shown with eNOS-deficient mice, which show impaired capacity to mobilize EPCs and impaired function of isolated EPCs.[175] Studies have demonstrated that colony-forming capacity and migratory function of circulating EPCs are impaired in conditions associated with reduced NO bioavailability, suggesting a link between eNOS activity and EPC function.[176, 177] The effects of HDL on circulating EPCs may also involve increasing cell survival and prevention of apoptosis. HDL prevents apoptosis of circulating EPCs through inhibition of caspase-3 activity.[178] Furthermore, administration of D-4F has been shown to cause EPCs to produce a robust increase in eNOS and heme oxygenase-1 thereby enhancing its survival which may contribute to vascular repair in diabetic rats.[125]

1.7.2 Alterations of the effects of HDL on endothelial repair processes in diabetes or CAD

Reconstituted HDL has been described to improve EOC availability in patients with type 2 diabetes, which were previously been shown to have reduced availability and impaired function of EOCs.[179] Meanwhile, HDL from patients with type 2 diabetes has recently been found to have a diminished capacity to stimulate endothelial cell proliferation, migration and adhesion to extracellular matrix and this impairment is associated with down regulation of SR-BI expression.[180] Notably, we have observed that HDL from patients with CAD has impaired endothelial repair capacity following vascular injury *in vivo* using nude mice carotid artery injury model.[16]

1.8 Effects of HDL on endothelial thrombotic function

1.8.1 Effects of HDL from healthy subjects on endothelial thrombotic function

Studies have shown that patients with arterial atherothrombosis have lower plasma levels of large HDL particles,[181-183] and an inverse relationship between the abundance of large HDL particles and the recurrence of adverse arterial events has been documented.[184] There has been some direct evidence demonstrating the anti-thrombotic actions of HDL. Infusion of reconstituted HDL limits the development of a procoagulant state in healthy volunteers given low doses of endotoxin.[185] In a rat model of acute

arterial thrombosis, infusion of apoA-I Milano caused a prolongation in the time of thrombus formation and a reduction in the weight of the thrombus.[186]

One potential mechanism contributing to the anti-thrombotic effects of HDL is the increased endothelial prostacyclin synthesis, which can modify thrombosis as well as other intravascular events. Prostacyclin acts synergistically with NO to induce vascular smooth muscle relaxation, inhibit platelet activation, and diminish the release of growth factors that stimulate the local proliferation of smooth muscle cells.[187] Incubation with native HDL increases prostacyclin production in cultured endothelial cells,[188, 189] and the effects can be recapitulated partially by delipidated HDL,[190] suggesting that both HDL-associated lipids and apolipoproteins are involved in the process. Prostacyclin release has also been shown to increase when isolated rabbit and rat hearts are infused with HDL.[191, 192] The impact of HDL on prostacyclin production in endothelium occurs by both the provision of arachidonate[188, 190, 193] and upregulation of Cox-2 expression.[194, 195] It has also been shown that HDL3 induces Cox-2 expression and prostacyclin release via a p38 MAP kinase/CREB-dependent pathway in endothelium.[195-198]. Recently, it was demonstrated that apoA-I, but not apoA-II, induced the expression of Cox-2 and the production of prostacylcin through the p38 MAPK, ERK1/2 and JAK2 pathways via ABCA1 in endothelial cells.[199] Involvement of SR-BI mediated PI3K-Akt-eNOS signaling in HDL-induced Cox-2 expression and prostacyclin release in endothelial cells has also been reported.[200]

Increased tissue factor and selectin expression on platelets and endothelial cells have been identified as critical factors in the initiation of thrombus formation. The phospholipid components of HDL have been demonstrated to contribute to the downregulation of E-selectin expression on endothelial cell surfaces.[9, 194] Furthermore, reconstituted HDL has been shown to downregulate thrombin-induced endothelial cell tissue factor expression in vitro.[201] HDL has also been shown to exert antithrombotic effects by inhibiting platelet activation. The administration of reconstituted HDL to humans or the infusion of apoA-I Milano into rats inhibits platelet aggregation.[185, 202] HDL may regulate platelet function by downregulating the release of platelet activating factor or by upregulating NO synthesis and release from endothelial cells.[195, 203] HDL also downregulates the biosynthesis of thromboxaneA2 (TxA2) and upregulates prostacyclin production which can decrease platelet aggregation as well as blunt leukocyte-endothelial cell interactions and thereby prevent the initiation and progression of atherogenesis.[204]

1.8.2 Alterations of the effects of HDL on endothelial thrombotic function in patients with diabetes

While there is no direct evidence showing that the anti-thrombotic actions of HDL may be impaired in inflammatory conditions, the involvement of PI3K-Akt-eNOS pathways[200] and also p38 MAPK, ERK1/2

and JAK2 pathways[195, 199] in HDL-induced Cox2 expression and prostacyclin release suggests that disruption in these pathways may likely lead to altered thrombotic actions of HDL on endothelial cells.

However, interestingly, a study showed that oxidized HDL strongly inhibits platelet activation and aggregation.[205] This anti-thrombotic action of oxidized HDL is mediated by SR-BI but independently of eNOS/Akt pathway.[205] More recently, HDL from patients with early stages of type 2 diabetes has been reported to upregulate Cox-2 expression and prostacyclin release in endothelial cells, mediated via S1P receptors.[206] The authors suggest that the increased in HDL-associated S1P levels in the early stage of type 2 diabetes may contribute to the anti-thrombotic actions of HDL indicating a compensatory protective mechanism during the early course of the disease.[206] More studies will be needed in this area to better understand the interaction between HDL and thrombotic pathways.

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1.10 Motivation of the PhD thesis

As highlighted in this chapter, plasma HDL cholesterol level is not an optimal therapeutic target. Given the heterogeneity of HDL particles, there needs to be a greater understanding of the quality and function of HDL, which may be mechanistically more important in relation to cardiovascular disease. It is increasingly evident that not all HDL particles are equally protective, and current clinical assays that simply measure the total quantity of HDL cholesterol fail to capture the qualitative functional differences. It remains unclear which function(s) of HDL are truly relevant for its cardiovascular effects. Furthermore, it also remains to be determined which alterations in the HDL particles that may account for its impaired vascular-protective properties. Therefore, there is a need for a better understanding of the mechanisms of action of HDL as well as a better characterization of the HDL particles. These goals form the foundation of my PhD thesis, the specific aims of which I elaborate further in detail below.

The first aim of my PhD thesis was to investigate the effects of HDL isolated from patients with CAD as compared to HDL from healthy subjects on endothelial function and to characterize the mechanisms leading to impaired vascular effects of HDL in patients with CAD, in particular the effects of HDL on endothelial apoptotic signaling pathways. Endothelial dysfunction, manifested as reduced endothelial nitric oxide availability, pro-inflammmatory activation and endothelial cell apoptosis, has been recognized as key mechanisms in the development and progression of atherosclerosis and other vascular disorders. The capacity of HDL to protect against endothelial dysfunction may therefore represent an important anti-atherogenic property of HDL. We found that HDL isolated from patients with CAD has impaired endothelial-protective capacity; findings that have consistently been reproduced also by other groups. More importantly, we found that this dysfunction is mediated via activation of specific signaling pathways in endothelial cells, suggesting that HDL from these patients carry a different sets of cargo that lead to the alterations in the vascular effects of HDL. This brings me to the second and third aims of my thesis.

As we learned that HDL isolated from patients with coronary disease is rather pro-inflammatory, it is of great interest to understand the effects of raising HDL from these patients. In order to test the HDL function hypothesis, we need to develop new metrics for assessing HDL function, instead of relying solely on HDL cholesterol level. The second aim of my thesis was to optimize cell-based assays that allow for relatively high-throughput assessment of the endothelial effects of HDL. Based on our earlier observations, we found that different endothelial effects of HDL may be accounted for by different components of HDL. Using these cell-based assays, we evaluated whether treatment that raise HDL cholesterol level may modulate the effects of HDL from patients with coronary disease on endothelial function.

Finally, in order to elucidate the mechanisms of action of HDL and its altered vascular effects in cardiovascular diseases, there is a need to characterize HDL cargo. The third aim of my thesis was to use targeted proteomics to investigate changes in the protein compositions of HDL that may modulate its function in coronary artery disease. The goal of this quantitative proteomics analysis of HDL was to identify novel candidate proteins that may provide new insights into the mechanisms of action of HDL and its alterations in cardiovascular disease.

Chapter 2

Altered activation of endothelial anti- and pro-apoptotic pathways by high-density lipoprotein from patients with coronary artery disease: Role of HDL-proteome remodeling

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Contribution by MR

Design of the study, experiments, data analysis, manuscript writing

2.1 Abstract

Endothelial dysfunction and injury are thought to play an important role in progression of coronary-artery-disease (CAD). High-density-lipoprotein from healthy subjects ($HDL_{Healthy}$) has been proposed to exert endothelial anti-apoptotic effects that may represent an important anti-atherogenic property of the lipoprotein. The present study therefore aimed to compare effects of HDL_{CAD} and $HDL_{Healthy}$ on activation of endothelial anti- and pro-apoptotic pathways and to determine which changes of the lipoprotein are relevant for these processes.

HDL was isolated from patients with stable CAD (HDL_{sCAD}), an acute coronary syndrome (HDL_{ACS}) and healthy subjects. $HDL_{Healthy}$ induced expression of the endothelial anti-apoptotic Bcl-2 protein Bcl-xL and reduced endothelial cell apoptosis *in vitro* and in apoE-deficient-mice *in vivo*. In contrast, HDL_{sCAD} and HDL_{ACS} did not inhibit endothelial apoptosis, failed to activate endothelial Bcl-xL and stimulated endothelial pro-apoptotic pathways, in particular p38-MAPK-mediated activation of the pro-apoptotic Bcl-2-protein tBid. Endothelial anti-apoptotic effects of $HDL_{Healthy}$ were observed after inhibition of endothelial nitric-oxide-synthase and after delipidation, but not completely mimicked by apoA-I or reconstituted HDL, suggesting an important role of the HDL-proteome. HDL proteomics analyses and subsequent validations and functional characterizations suggested a reduced clusterin- and increased apoC-III-content of HDL_{sCAD} and HDL_{ACS} as mechanisms leading to altered effects on endothelial apoptosis.

In this chapter, I will present a study that demonstrates for the first time that HDL_{CAD} does not activate endothelial anti-apoptotic pathways, but rather stimulates potential endothelial pro-apoptotic pathways. HDL-proteome remodeling plays an important role for these altered functional properties of HDL. These findings provide novel insights into mechanisms leading to altered vascular effects of HDL in coronary disease.

2.2 Introduction

Reduced plasma levels of HDL cholesterol are associated with an increased risk of coronary artery disease (CAD).[1] Moreover, in patients with CAD that are treated with statin and have low levels of low-density lipoprotein (LDL) cholesterol, reduced HDL cholesterol levels were predictive of major cardiovascular events.[2] Besides promoting reverse cholesterol transport,[3, 4] HDL has been demonstrated to exert anti-atherosclerotic effects, including anti-inflammatory properties and stimulation of endothelial nitric oxide (NO) production.[5-9] However, these effects of HDL have been observed to be highly heterogenous in patients with CAD or diabetes.[10-12]

Endothelial dysfunction and injury are thought to contribute importantly to the progression of CAD.[13-15] Experimental studies have indicated that atherosclerotic lesion-prone vascular regions are characterized by a high endothelial cell turn-over,[16] which has been attributed to an increased rate of endothelial cell apoptosis. Moreover, superficial atherosclerotic plaque erosion with loss of an intact endothelial cell monolayer is observed quite frequently in patients with an acute coronary syndrome (ACS) based on pathological[17, 18] as well as on observations from clinical high-resolution intracoronary imaging studies.[19] In pathological studies of coronary atherosclerotic plaque erosion thrombi were observed in direct contact with the intima in areas with absent endothelium likely promoting disease progression.[17] Endothelial cell apoptosis has therefore been suggested to contribute importantly to the pathophysiology of coronary disease.[20-22] The capacity of HDL to attenuate endothelial cell apoptosis may therefore represent a potentially important anti-atherogenic property of HDL.[23-26]

However, as described above, the vascular effects of HDL can be highly variable in patients with cardiovascular disease, a phenomenon referred to as "HDL dysfunction".[10, 12, 27] The present study was therefore designed to compare the effects of HDL from patients with stable CAD or an ACS and healthy subjects on endothelial anti-apoptotic and pro-apoptotic signaling pathways. Moreover, we aimed to characterize alterations in HDL from patients with CAD that may lead to altered functional properties of HDL with respect to activation of endothelial anti-apoptotic and pro-apoptotic signaling pathways.

2.3 Methods

An expanded description of the methods is available in Supplementary Methods.

Patient Characteristics. Patients with stable CAD or an ACS (STEMI or NSTEMI) and healthy subjects (without cardiovascular risk factors) were recruited at the University Hospital of Zurich. Exclusion criteria were accompanying infectious, inflammatory or autoimmune disorders, advanced kidney or liver failure, diabetes, neoplastic disorders and a history of major surgery or trauma within the previous month.

Isolation of High-Density Lipoprotein and HDL-delipidation. HDL was isolated by either sequential ultracentrifugation (*d*=1.063-1.21g/ml) or gel filtration chromatography. For proteomics studies and functional studies HDL was delipidated using methanol/chloroform extraction. Detailed description of the methods is provided in Supplementary Methods.

Measurement of Endothelial Cell Apoptosis *In Vitro*. Endothelial apoptosis was measured by FACS analysis with annexin-V-staining or TUNEL assay, as well as using fluorescence microscopy with annexin-V-staining. Caspase-3 activity was measured using a colorimetric assay. Detailed description of the methods is provided in Supplementary Methods.

Measurement of Endothelial Cell Apoptosis *In Vivo* using FACS Analysis and Active Caspase-3 Staining. Male apoE(-/-).C57BL/6 mice, aged 12-16 weeks, were used for tail-vein injection of HDL (14mg HDL-protein/kg body-weight), following anaesthesia with inhalation of isoflurane(3%). Twenty-four hours after the injection of HDL or PBS-buffer, mice were euthanized and the aorta were harvested and immediately digested for FACS analysis of endothelial cell apoptosis or fixated for histological staining. For detailed description of the methods, please refer to the Supplementary Methods.

Biochemical Validation of HDL-associated Proteins. ELISA was used for validation of changes in clusterin-(BioVendor R&D, USA) and apoC-III-(AssayPro, USA) content of HDL.

Western Blot analysis. Phosphorylation of Akt at serine residue473, phosphorylation of MAPK-p38 and expression of Bcl-2, Bcl-xL, tBid, Bak were determined by western blot analysis. Anti-human phospho-Akt(Ser473), anti-human total Akt, anti-human phospho-p38-MAPK, anti-human total p38-MAPK were purchased from Cell Signaling Technology. Antibodies against human Bcl2, Bcl-xL, tBid and Bak were from Cell Signaling Technology.

Statistical Analysis. All data are expressed as mean±SEM. All analyses were performed with SPSS 21.0 (IBM SPSS). Significance was tested using Student's-*t*-test and ANOVA with Dunnett *post-hoc* test for multiple comparison analysis. A value of $p<0.05$ was considered statistically significant.

2.4 Results

HDL was isolated from patients with coronary disease (HDL_{CAD}), with either stable CAD (HDL_{sCAD}) or an acute coronary syndrome (HDL_{ACS}), and age- and gender-matched healthy subjects ($HDL_{Healthy}$). The characteristics of the study population are shown in Table 2.1.

Table 2.1 Characteristics of the study population.

Characteristics	Healthy subjects n=20	Stable coronary artery disease n=20	Acute coronary syndrome n=20	P-value
Demographics				
Age, mean (years)	58±5	59±7	61±6	n.s
Sex (male/female)	12/8	14/6	11/9	
BP systolic, mean (mm Hg)	119.9±8.7	127.2±17.3	121.2±16.4	n.s
BP diastolic, mean (mm Hg)	76.6±9.1	77.9±8.4	71.6±10.5	n.s
BMI, mean (kg/m^2)	23±2	27±3	26±4	<0.05
MAP, mean (mm Hg)	91.0±8.0	94.3±9.4	88.1±11.7	n.s
Laboratory parameters				
Fasting glucose (mmol/l)	5.6±1.3	5.9±1.4	6.3±1.3	n.s
HbA1c (in %)	5.7±0.3	5.8±0.8	5.9±0.4	n.s
Total cholesterol (mmol/l)	5.8±0.8	4.3±0.7	4.7±1.3	<0.05
HDL cholesterol (mmol/l)	1.9±0.5	1.3±0.3	1.3±0.3	<0.05
LDL cholesterol (mmol/l)	3.2±0.7	2.4±0.6	3.0±1.2	n.s
Triglyceride (mmol/l)	0.9±0.4	1.3±0.5	1.2±0.6	n.s
CRP (µmol/l)	0.8±0.4	2.8±4.9	4.5±4.9	<0.05
Creatinine (µmol/l)	75.6±13.3	86.6±26.0	73.1±14.3	n.s
Medications				
Statins (in %)	0	90	75	
Beta blocker (in %)	0	70	50	
Diuretics (in %)	0	35	20	
ACE-I/ARB (in %)	0	75	75	
Calcium blocker	0	10	5	
Aspirin (in %)	0	95	100	
Clopidogrel (in %)	0	60	60	

Abbreviations: BP, blood pressure; BMI, body mass index; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.
Reported P values are from one-way ANOVA.

2.4.1 Effects of $HDL_{Healthy}$ and HDL_{CAD} on endothelial cell apoptosis *in vitro* and *in vivo*

$HDL_{Healthy}$ markedly reduced endothelial cell apoptosis *in vitro*, whereas in marked contrast, HDL_{sCAD} or HDL_{ACS} did not limit endothelial apoptosis induced by serum and growth factor deprivation (Figures

2.1A-C,E-F). Endothelial caspase-3 activity was reduced by $HDL_{Healthy}$, but not after treatment with HDL_{sCAD} or HDL_{ACS} (Figure 2.1D). Similarly, detection of fragmented DNA using TUNEL staining showed significantly attenuated endothelial apoptosis by $HDL_{Healthy}$ (26.7%±12.0% vs control 41.6%±10.3%, $p<0.05$), but not HDL_{sCAD} or HDL_{ACS} (36.9%±9.1% and 32.2%±13.6%, respectively). Furthermore, $HDL_{Healthy}$ but not HDL_{sCAD} or HDL_{ACS} reduced endothelial apoptosis induced by another stimulus, i.e. TNF-α, as measured by annexin-V staining (Figure 2.1G) or TUNEL assay (apoptotic endothelial cells after $HDL_{Healthy}$ vs. HDL_{CAD} 23.02%±14.4% vs. 39.7%±13.7%; $p<0.05$). The rate of TNF-α-induced endothelial apoptosis was similar as compared to previous studies, such as the study by Spyridopoulos et al.[28] reporting 39.3% apoptotic endothelial cells after TNF-α exposure.

Moreover, we examined the capacity of HDL to impact on endothelial apoptosis *in vivo* by injecting isolated HDL to apoE-/- mice. Administration of $HDL_{Healthy}$ (14mg HDL-protein/kg body weight) to apoE-/- mice reduced endothelial apoptosis in the aorta (harvested after 24hours) while HDL_{CAD} did not reduce endothelial apoptosis as determined by Annexin-V measurement as well as immunofluorescence staining with TUNEL and active caspase-3 assay (Figure 2.2A-D). In these studies, we did not observe a significant difference between the anti-apoptotic capacity of HDL_{sCAD} as compared to HDL_{ACS} (7.2±3.1% vs 6.3±3.8%; $p=0.51$).

2.4.2 $HDL_{Healthy}$ reduces endothelial apoptosis after inhibition of endothelial NO-synthase

Endothelial NO synthase is a potentially important regulator of endothelial apoptosis and effects of HDL on eNOS are adversely affected in patients with CAD.[10, 29] We therefore examined whether the effects of HDL on endothelial cell apoptosis are critically dependent on eNOS. Notably, inhibition of eNOS-mediated NO production by both, pharmacological inhibition with N-nitro-L-arginine methyl ester (L-NAME) and eNOS-specific RNA interference did not prevent endothelial anti-apoptotic effects of $HDL_{Healthy}$ (Figure 2.3A), suggesting that HDL activates endothelial anti-apoptotic pathways independent of eNOS.

2.4.3 Role of protein moiety for endothelial anti-apoptotic capacity of $HDL_{Healthy}$

To investigate which alterations of the HDL particle may impair the capacity to limit endothelial apoptosis, we examined effects of the HDL protein fraction (delipidated HDL), rHDL, purified apoA-I and recombinant apoA-I on endothelial apoptosis. Interestingly, delipidated $HDL_{Healthy}$ exerted a potent endothelial anti-apoptotic activity as measured by annexin-V staining (Figure 2.3B). The endothelial anti-apoptotic effects of delipidated $HDL_{Healthy}$ were more profound as compared to purified or recombinant apoA-I or rHDL (Figure 2.3B), suggesting an important role of the HDL proteome and its remodeling for altered effects of HDL_{CAD} on endothelial apoptosis. Similarly, by using TUNEL staining we observed that $HDL_{Healthy}$ and delipidated $HDL_{Healthy}$ significantly reduced the number of apoptotic endothelial cells

(22.2%±8.0% and 19.0%±14.1% vs control 37.6±10,2%, p<0.05), that was not observed with apoA-1 or rHDL alone (29.0%±8.7% and 25.3%±10.4%, respectively, p=0.11 and p=0.08 vs. control).

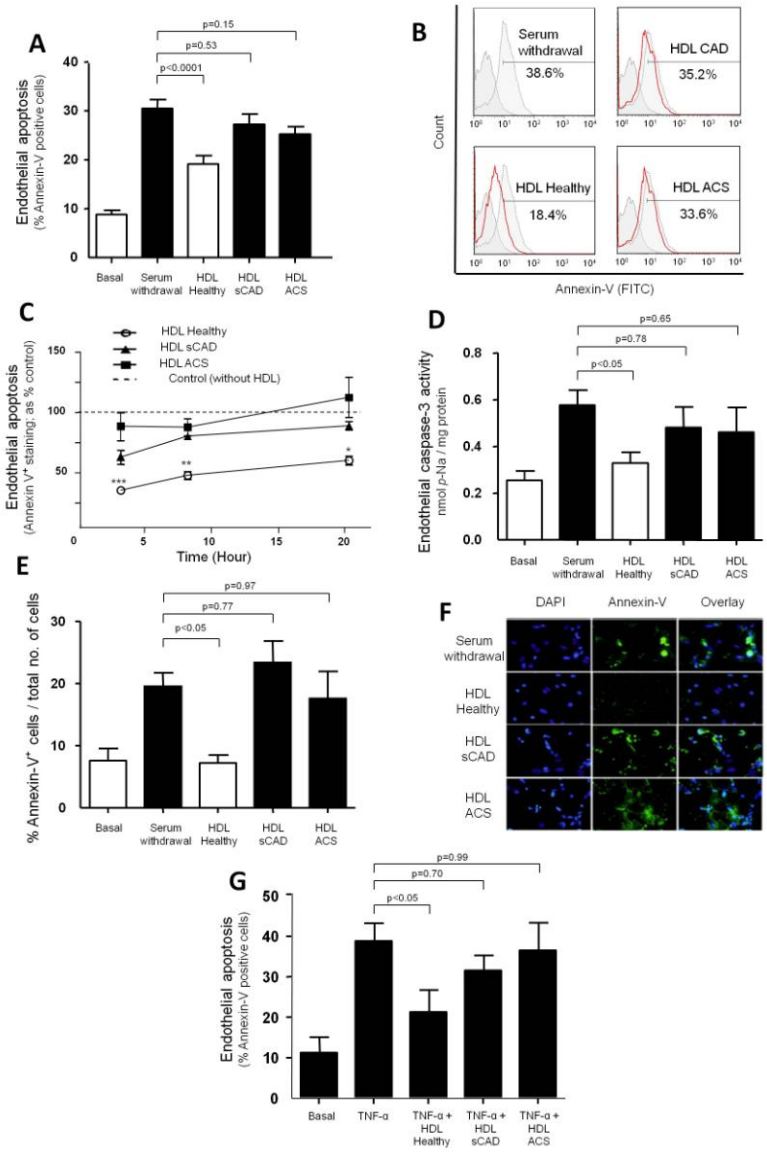

Figure 2.1 Effects of $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} on endothelial apoptosis *in vitro*. (A) The impact of $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} (50µg/ml based on protein measurement) on endothelial apoptosis was determined by annexin-V staining using FACS analysis after 16 hours of serum deprivation (n=20 per group) (B) Representative examples of flow cytometry analyses of annexin-V staining. Dark grey solid line: control cells for autofluoresence, Light grey dotted line: serum withdrawal, Red line: HDL-treated cells with serum withdrawal. (C) Endothelial apoptosis in the absence or presence of HDL (50µg/ml) was determined for the indicated time intervals following serum/growth factor deprivation. The dotted line represents buffer-treated cells with serum withdrawal. (D) Caspase-3 activity was measured from the lysate of endothelial cells treated with or without HDL (50µg/ml; n=20 per group). (E,F) Fluorescence microscopy of apoptotic endothelial cells stained with annexin-V-FITC. Number of annexin-V+ cells was counted per high power field (average values were taken from 5 hp-fields). (G) Effect of

$HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} (50µg/ml) on endothelial cell apoptosis induced by TNF-α as measured by annexin-V staining using FACS analysis.

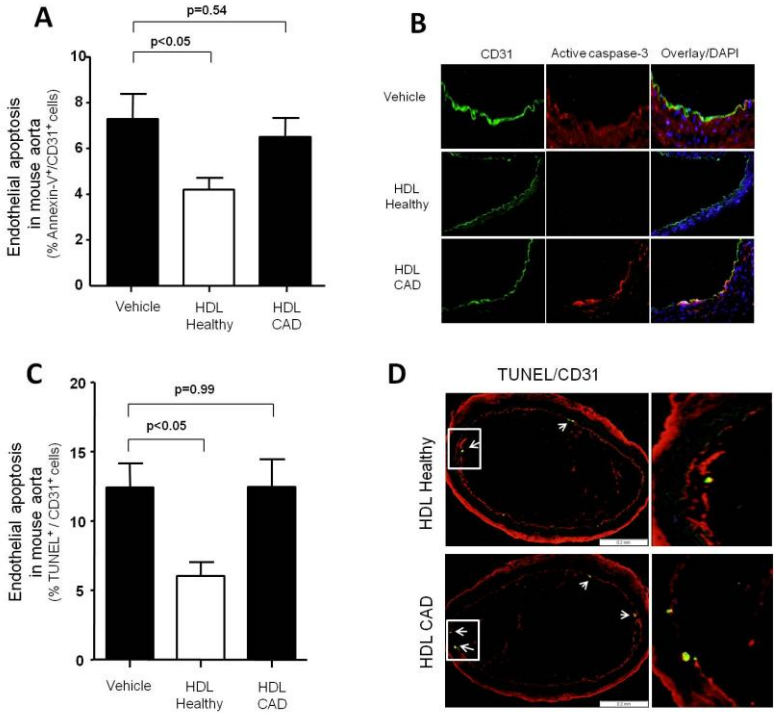

Figure 2.2 Effects of $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} on endothelial apoptosis *in vivo*. (A) Administration of $HDL_{Healthy}$, but not HDL_{CAD} (14mg HDL protein/kg body weight) to ApoE-/- mice via tail-vein injection reduced endothelial apoptosis as detected in the aorta after 24 hours (n=8-9 per group) as measured by co-staining of annexin-V+ and CD31+ using FACS analysis. (B) Immunofluorescence staining showed reduced active caspase-3 staining of endothelial cells of mouse aortic sections after treatment with $HDL_{Healthy}$, but not with $HDL_{CAD.}$ (C, D) TUNEL-FITC staining of mouse aortic sections co-stained with CD31-Texas-red. Number of TUNEL+ cells / Number of CD31+ cells was counted per high power field and the average was taken from 5 fields.

2.4.4 Proteomics analysis comparing $HDL_{Healthy}$ and HDL_{CAD} using LC-ESI-MS/MS

We therefore used a mass spectrometric-based approach to identify changes in HDL-associated proteins in coronary disease. The spectral index was used for relative quantification of each protein of interest,[30, 31] as described in detail in the Supplementary Material. Based on this approach, we identified 78 HDL-associated proteins with substantial differences in their quantitative abundance between $HDL_{Healthy}$ and HDL_{CAD} (Figure 2.3C, Supplementary Table 2.1). We further applied gene ontology analysis to identify proteins that are related to regulation of apoptosis. Based on these analyses two proteins, clusterin and apoC-III, were selected for further assessment of their potential relevance in mediating effects of HDL on

endothelial apoptosis, given their differential abundance in HDL_{CAD} and their potential role in regulation of apoptotic processes.

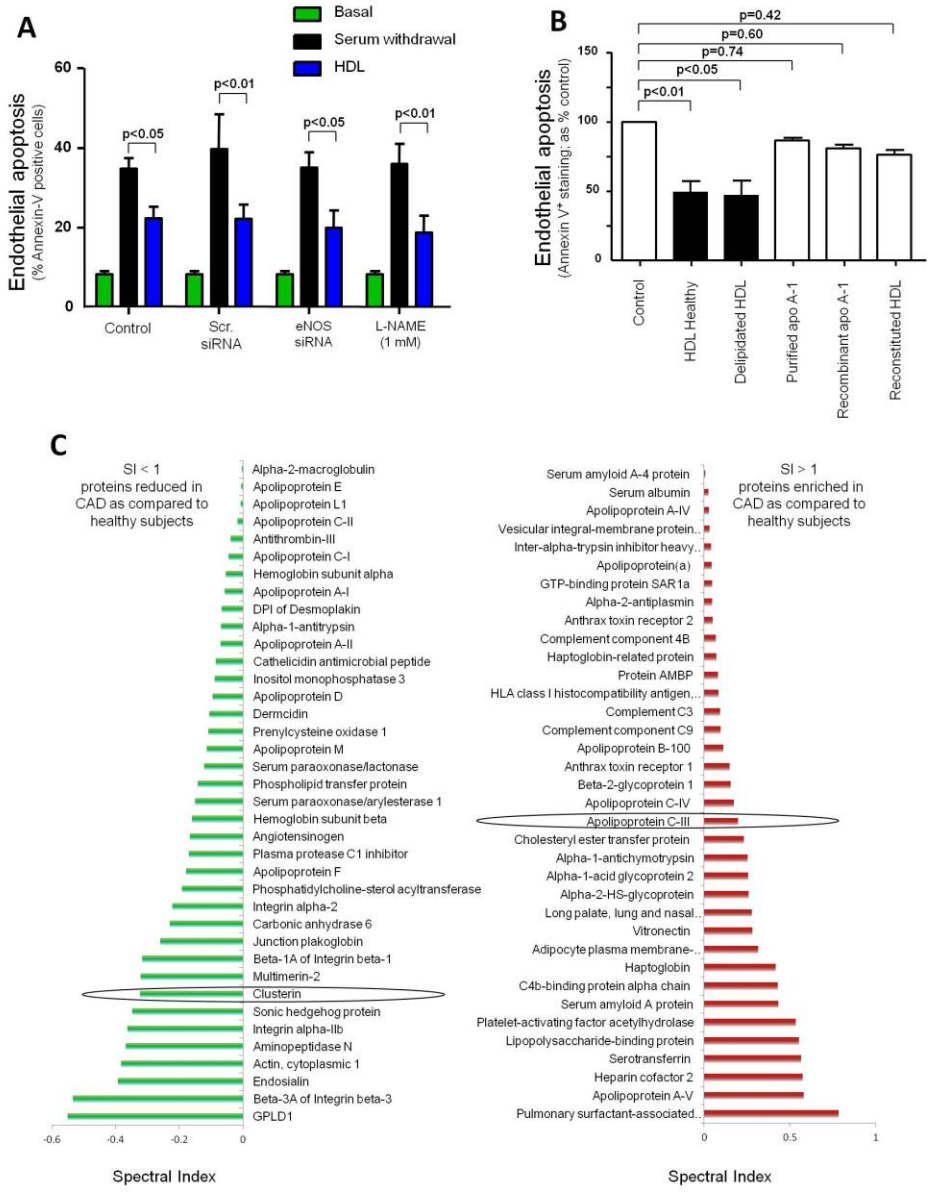

Figure 2.3 Evaluation of the role of endothelial NO synthase and the effects of different HDL components ($HDL_{Healthy}$) on endothelial apoptosis (A) $HDL_{Healthy}$ reduced endothelial apoptosis after inhibition of eNOS-mediated endothelial NO production by siRNA knockdown of *eNOS* or treatment with L-NAME (1 mM) (n=6-8 per group). Scr: scrambled. (B) Effects of $HDL_{Healthy}$ (50µg/ml), delipidated HDL (50µg/ml), apo A-1 (50µg/ml) or reconstituted HDL (50µg/ml) on endothelial apoptosis as measured by FACS analysis with annexin-V staining (n=6 per group). Reconstituted HDL was prepared with the

sodium cholate dialysis method using an apoA-I/POPC/cholesterol molar ratio of 1:80:10. (C) LC-ESI-MS/MS analysis of $HDL_{Healthy}$ and HDL_{CAD} (n=6 per group). Proteins identified were quantified using spectral index and data are presented as proteins reduced or enriched in HDL_{CAD}.

2.4.5 Validation of altered clusterin levels in HDL_{CAD} as compared to $HDL_{Healthy}$ and its relevance for endothelial anti-apoptotic effects of HDL

Proteomics analysis indicated reduced clusterin levels in HDL_{CAD} relative to $HDL_{Healthy}$ (spectral index of -0.323) (Supplementary Table 2.1). An MS/MS fragmentation pattern of a proteotypic peptide of clusterin is shown in Figure 2.4A. To further validate this finding, we quantified clusterin in $HDL_{Healthy}$ and HDL_{CAD} isolated by two different methods, i.e. ultracentrifugation and gel filtration. Independent of the isolation method, substantially lower clusterin levels were detected in HDL_{sCAD} and HDL_{ACS} as compared to $HDL_{Healthy}$ (Figure 2.4B, Table 2.2).

To further investigate a potential functional role of clusterin in HDL for effects on endothelial apoptosis, we pre-incubated $HDL_{Healthy}$ with specific clusterin blocking antibodies (Santa Cruz Biotechnology, USA) and supplemented HDL_{CAD} with clusterin. Pre-incubation of $HDL_{Healthy}$ with clusterin-blocking antibodies (1:50 or 1:100 dilution), but not with an IgG isotype control, reduced endothelial anti-apoptotic effects of $HDL_{Healthy}$ in a dose-dependent manner as measured by annexin-V staining (Figure 2.4C). The presence of the clusterin-blocking antibody decreased the capacity of $HDL_{Healthy}$ to reduce endothelial cell apoptosis as measured by caspase-3 activity (Figure 2.4D) and TUNEL staining (apoptotic endothelial cells: 38.2±5.6% versus 23.1±6.2%, $p<0.05$). Similarly, the capacity of $HDL_{Healthy}$ to attenuate TNF-α-induced endothelial apoptosis was reduced in the presence of clusterin blocking antibody (Figure 2.4E). Vice versa, supplementation of HDL_{CAD} with purified clusterin increased its endothelial anti-apoptotic effects (Figure 2.4F). Notably, purified clusterin alone had no significant effect on endothelial apoptosis, indicating that binding of clusterin to the HDL particle was important for its full endothelial anti-apoptotic effects. To further investigate the role of HDL-associated clusterin for endothelial anti-apoptotic effects of HDL, we prepared reconstituted HDL with and without clusterin. Addition of clusterin to rHDL resulted in a more profound endothelial anti-apoptotic effect (Figure 2.4G), supporting a role of HDL-bound clusterin for endothelial anti-apoptotic properties of $HDL_{Healthy}$. Of note, corresponding amounts of HDL-bound clusterin after supplementation of reconstituted HDL or HDL_{CAD} were within a similar range as compared to clusterin levels observed in $HDL_{Healthy}$, further supporting the concept that reduced levels of HDL-bound clusterin in HDL_{CAD} impact on the capacity of HDL to limit endothelial apoptosis (Supplementary Figure 2.1A).

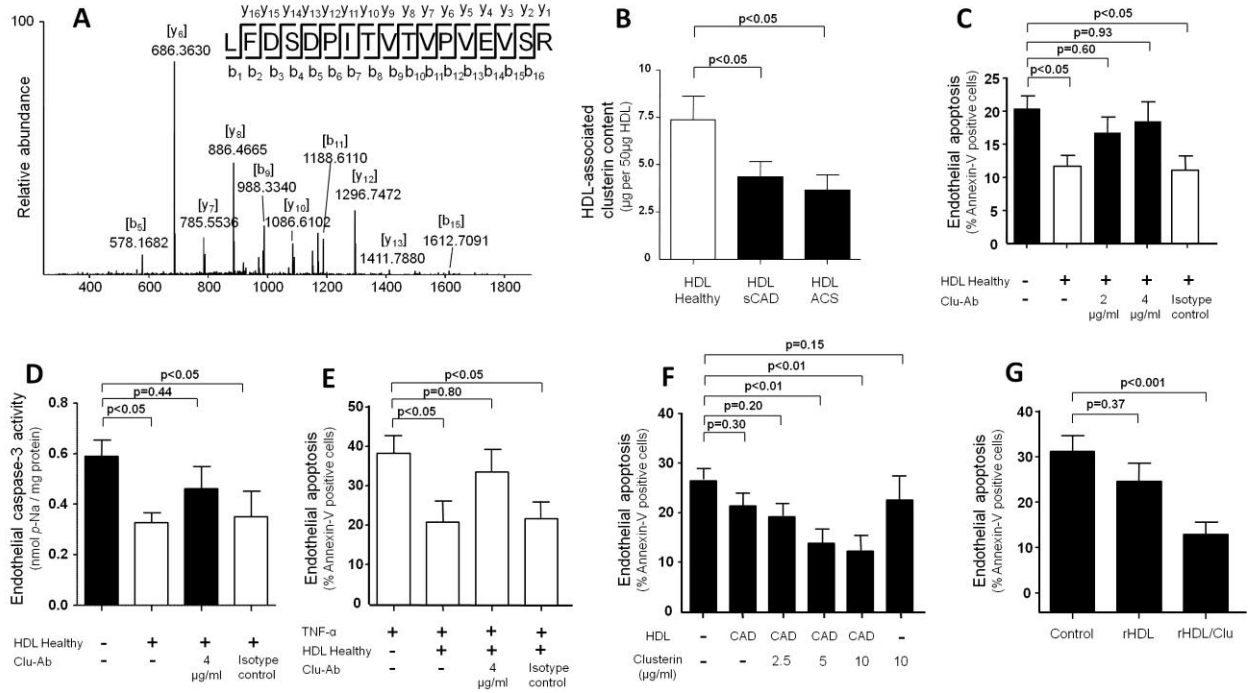

Figure 2.4 Role of HDL-associated clusterin for effects of $HDL_{Healthy}$ on endothelial anti-apoptotic pathways. (A) MS/MS spectrum of a proteotypic peptide of clusterin. (B) Concentration of clusterin associated with $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} **as** quantified using ELISA. (C) $HDL_{Healthy}$ (50µg/ml) was pre-incubated with specific blocking antibody against clusterin (2-4µg/ml) and the effects of HDL on endothelial cell apoptosis induced by serum withdrawal were analyzed using annexin-V staining by FACS analysis, and (D) caspase-3 activity assay. (E) Effect of the clusterin blocking antibody on the anti-apoptotic capacity of $HDL_{Healthy}$ (50µg/ml) as examined in TNF-α stimulated endothelial cells. (F) Effects of HDL_{CAD} (50µg/ml) on endothelial cell apoptosis in the presence of increasing concentrations of purified human clusterin (2.5-10µg/ml) were determined. (G) Effects of reconstituted HDL (50µg/ml) in the absence or presence of clusterin on endothelial cell apoptosis were analyzed.

2.4.6 Increased apoC-III levels in HDL_{sCAD} and HDL_{ACS} and relevance for effects of HDL on endothelial apoptosis

Based on the spectral index of the proteomics analysis, levels of apoC-III were increased in HDL_{sCAD} and HDL_{ACS} as compared to $HDL_{Healthy}$ (spectral index of 0.197) (Supplementary Table 2.1). An MS/MS fragmentation pattern of a proteotypic peptide of apoC-III is shown in Figure 2.5A. To further validate this finding we quantified apoC-III in $HDL_{Healthy}$ and HDL_{CAD} isolated by either ultracentrifugation or gel filtration. HDL_{sCAD} and HDL_{ACS} were enriched with apoC-III as compared to $HDL_{Healthy}$ (Figure 2.5B, Table 2). Pre-incubation of HDL_{CAD} with a specific blocking antibody for apoC-III (1:50 dilution), but not with an isotype-control-antibody, improved its capacity to reduce endothelial apoptosis, both after serum withdrawal (Figures 5C-F) or TNF-α-exposure (Figures 2.5G-I). Similarly, the blocking antibody against apoC-III increased the endothelial anti-apoptotic capacity of HDL_{CAD} as measured by TUNEL staining (25.2±5.6% versus HDL_{CAD} alone 37.1±6.2%, $p<0.05$). Conversely, pre-incubation of $HDL_{Healthy}$ with purified apoC-III impaired its capacity to attenuate endothelial apoptosis (Figure 2.5J). The corresponding

amounts of HDL-bound apoC-III after supplementation of $HDL_{Healthy}$ with apoC-III were in a similar range as compared to HDL-bound apoC-III levels observed in HDL_{CAD} (Supplementary Figure 2.1B).

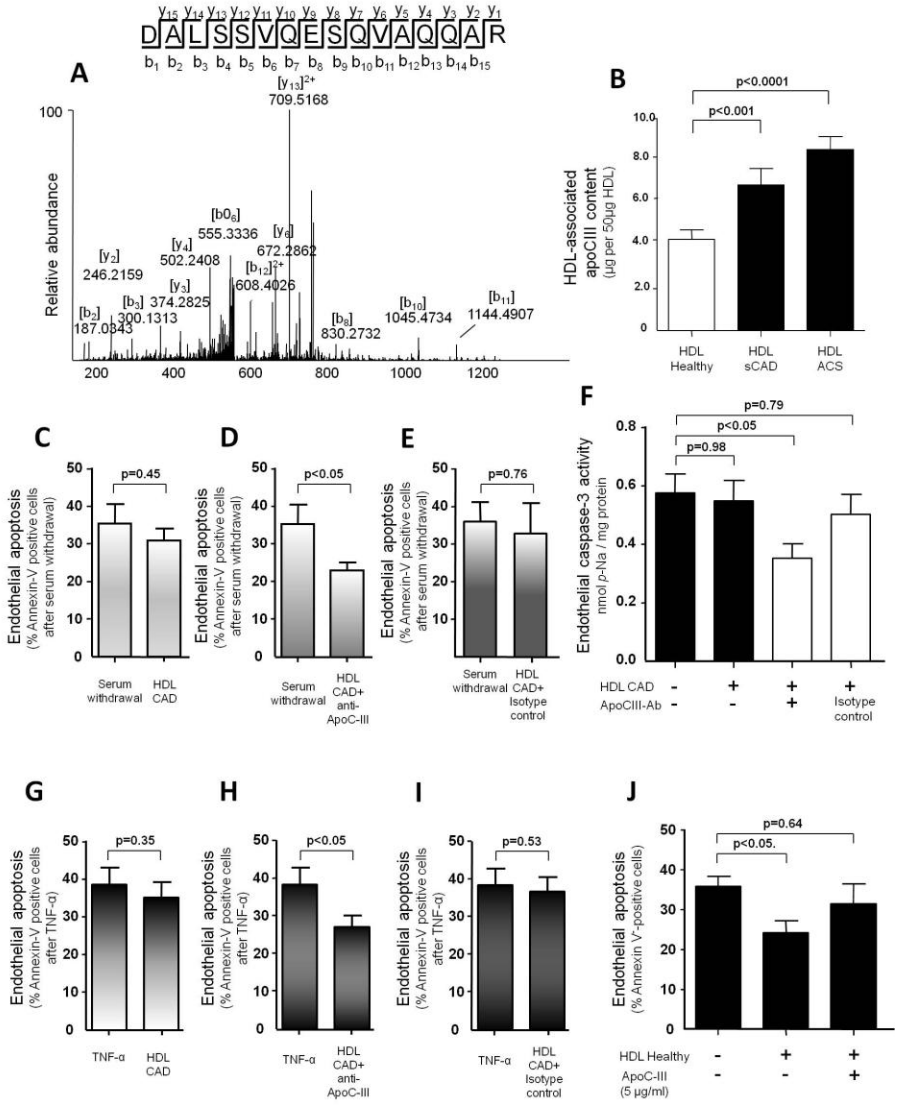

Figure 2.5 Role of HDL-associated apoC-III for impaired endothelial anti-apoptotic effects of HDL in patients with CAD. (A) MS/MS spectrum of a proteotypic peptide of apoC-III. (B) The amount of apoC-III in $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} was quantified with ELISA. (C,D,E) HDL_{CAD} (50µg/ml) was pre-incubated with specific blocking antibody against apoC-III (20µg/ml or 1:50 dilution) or isotype control and the effects of HDL on serum withdrawal induced- endothelial apoptosis were analyzed with annexin-V staining using FACS analysis and (F) caspase-3 activity measurement. (G,H,I) Effects of HDL_{CAD} pre-incubated with a specific blocking antibody against apoC-III or isotype control on TNF-α-induced-endothelial apoptosis were analyzed with annexin-V staining using FACS analysis. (J) Effects of $HDL_{Healthy}$ (50µg/ml) on endothelial cell apoptosis in the presence of purified human apoC-III (5µg/ml).

2.4.7 $HDL_{Healthy}$ activates the endothelial anti-apoptotic Bcl-2 protein Bcl-xL via PI3K/Akt pathway, whereas HDLCAD upregulates the endothelial pro-apoptotic Bcl-2 protein tBid via MAPK-p38 – role of HDL-associated clusterin and apoC-III.

Further assessment of signaling mechanisms involved in effects of HDL on endothelial apoptosis showed that the anti-apoptotic capacity of $HDL_{Healthy}$ was mediated via PI3K/Akt, since PI3K inhibition by wortmannin or LY294002 prevented endothelial anti-apoptotic effects of $HDL_{Healthy}$, whereas these anti-apoptotic pathways were not activated by HDL_{CAD} (Figure 2.6A). $HDL_{Healthy}$ phosphorylated endothelial Akt at Ser473, which was not observed using HDL_{sCAD} or HDL_{ACS} (Supplementary Figure 2.2A).

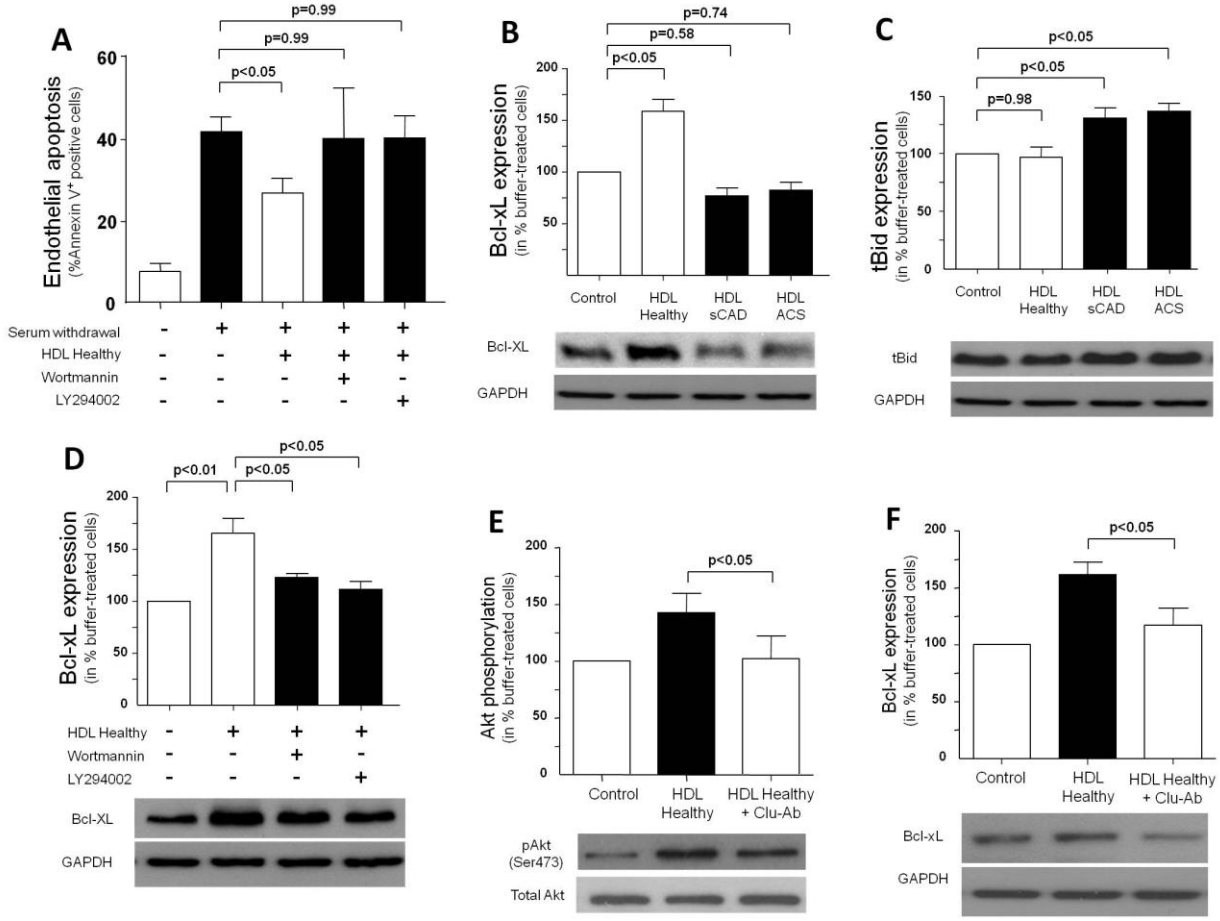

Figure 2.6 Activation of the endothelial anti-apoptotic signaling pathways by $HDL_{Healthy}$. (A) Pre-treatment of endothelial cells with PI3K inhibitors, wortmannin (100nM) or LY294002 (50µM) reversed the anti-apoptotic effects of $HDL_{Healthy}$ (50µg/ml). (B) Effects of $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} (50µg/ml) on the expression of endothelial Bcl-xL expression and (C) endothelial tBid expression were assessed by western blot analysis (n=6 per group). (D) Effects of $HDL_{Healthy}$ (50µg/ml) on Bcl-xL expression in HAECs pre-treated with PI3K inhibitor as analyzed by western blot analysis. (E) Effects of $HDL_{Healthy}$ pre-incubated with specific blocking antibody against clusterin (4µg/ml) on the phosphorylation of Akt at Ser473, (F) Bcl-xL expression (n=6 per group), as detected by western blot analysis.

Our further analysis of downstream targets of HDL on endothelial apoptotic pathways revealed a differential regulation of anti-apoptotic and pro-apoptotic proteins from the Bcl-2 family by $HDL_{Healthy}$ and HDLCAD. $HDL_{Healthy}$ activated Bcl-xL, an anti-apoptotic Bcl-2 protein[32, 33] whereas HDL_{CAD} activated tBid, a pro-apoptotic Bcl-2 protein[34, 35] (Figures 2.6B and 2.6C). No significant differences were observed on the expression of other Bcl-2 proteins, anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bak by $HDL_{Healthy}$ and HDL_{CAD} (*data not shown*). Wortmannin or LY294002 inhibited effects of $HDL_{Healthy}$ on Bcl-xL expression (Figure 2.6D). Notably, pre-incubation of $HDL_{Healthy}$ with clusterin blocking antibody reduced HDL-dependent phosphorylation of Akt at Ser473 (Figure 2.6E) and impaired upregulation of Bcl-xL expression (Figure 2.6F). These findings indicate that clusterin contributes to effects of $HDL_{Healthy}$ on stimulation of PI3K/Akt which in turn modulates endothelial expression of the anti-apoptotic Bcl-xL.

Furthermore, assessment of the role of mitogen-activated protein kinase (MAPK) demonstrated that HDL_{CAD} increased endothelial phosphorylation of p38-MAPK, whereas $HDL_{Healthy}$ had no effect (Figure 2.7A). Inhibition of HDL_{CAD} induced p38-MAPK-activation using the inhibitor SB203580 blocked the increase in endothelial expression of tBid (Figure 2.7B). Pre-incubation of HDLCAD with the specific blocking antibody against apoC-III inhibited the increased phosphorylation of p38 MAPK (Figure 2.7C) and the increased expression of tBid (Figure 2.7D). These observations suggest that HDL-associated apoC-III stimulates pro-apoptotic signaling by phosphorylation of p38-MAPK and upregulation of endothelial expression of pro-apoptotic tBid.

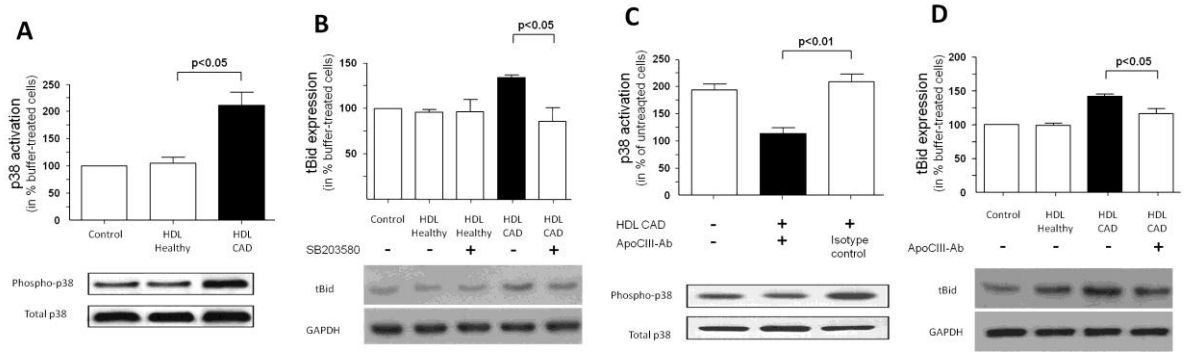

Figure 2.7 Stimulation of the endothelial pro-apoptotic signaling pathways by HDL_{CAD}. (A) Effects of $HDL_{Healthy}$ and HDL_{CAD} on phosphorylation of MAPK p38 were analyzed by western blot analysis (n=8-10 per group). (B) Effects of HDL_{CAD} on endothelial tBid expression pre-incubated with SB203580 (10μM). (C) Effects of HDL_{CAD} pre-incubated with specific blocking antibody against apoC-III on the phosphorylation of MAPK p38, (D) tBid expression (n=6-8 per group), as detected by western blot analysis.

2.4.8 Assessment of clusterin and apoC-III content in other lipoprotein fractions

The content of clusterin in serum or in the LDL/VLDL fraction was not reduced in patients with CAD as compared to healthy subjects (Table 2.2), whereas clusterin levels were substantially lower in HDL_{CAD} as compared to $HDL_{Healthy}$ isolated by both ultracentrifugation or gel filtration methods, compatible with the concept of a reduced binding of clusterin to HDL_{CAD} (Table 2.2).

The apoC-III content was mainly increased in HDL_{CAD}, but was also to some extent elevated in serum and the LDL/VLDL fraction of patients with CAD as compared to healthy subjects using both, ultracentrifugation or gel filtration lipoprotein isolation protocols (Table 2.2). These observations suggest a systemic increase of apoC-III levels in patients with CAD.

Table 2.2 Concentrations of apolipoprotein C-III and clusterin in serum and lipoprotein fractions of patients with CAD and healthy subjects.

	Healthy (n=12)	CAD (n=12)	P-value
Apolipoprotein C-III, mg/l			
Serum	129.8±23.3	180.4± 33.5	0.0003
UC-HDL	84.7±17.8	112.6±28.8	0.0092
UC-LDL/VLDL	35.7±20.2	62.5±18.2	0.0025
FPLC-HDL	88.0±29.7	114.2±26.7	0.0277
FPLC-LDL/VLDL	39.4±18.6	66.5±22.1	0.0037
Clusterin, mg/l			
Serum	77.6±13.5	78.6±23.3	0.91
UC-HDL	56.5±11.7	38.3±11.7	0.0026
UC-LDL/VLDL	16.6±7.1	24.1±8.0	0.0244
FPLC-HDL	59.0±23.0	40.4±14.6	0.0439
FPLC-LDL/VLDL	16.9±8.1	25.1±12.2	0.0876

UC refers to lipoprotein preparation with ultracentrifugation. FPLC refers to lipoprotein separation with fast protein liquid chromatography. Data are expressed as mean ± SD. P-values were determined by Student's-*t*-test.

2.5 Discussion

In the present study we have for the first time compared the effects of HDL from healthy subjects and from patients with CAD on endothelial cell anti- and pro-apoptotic signaling pathways. Importantly, while $HDL_{Healthy}$ substantially reduced endothelial cell apoptosis *in vitro* and *in vivo*, no such effects were observed for HDL from patients with sCAD or ACS.

Of note, our studies revealed differential effects of $HDL_{Healthy}$ and HDL_{CAD} on endothelial anti- and pro-apoptotic signaling pathways, in particular on members of the Bcl-2 family of proteins, that are critical regulators of apoptosis. $HDL_{Healthy}$, but not HDL_{CAD}, activated the endothelial anti-apoptotic Bcl-xL pathway. In contrast, HDL_{CAD} activated endothelial tBid, a pro-apoptotic Bcl-2 protein. Our studies further suggest that differences in the proteome of HDL_{CAD}, in particular reduced HDL-associated clusterin and increased HDL-associated apoC-III, play an important role for altered activation of endothelial anti- and pro-apoptotic signaling pathways (Figure 2.8).

Endothelial dysfunction and injury are thought to play an important role in initiation and progression of atherosclerotic CAD.[13-15] Experimental studies have shown that atherosclerotic lesion-prone vascular regions such as bifurcations are characterized by high endothelial cell turn-over,[16] likely indicating an increased rate of endothelial cell apoptosis.[22] Furthermore, coronary atherosclerotic plaque erosion with loss of an intact endothelial cell monolayer is frequently observed in patients with an acute coronary syndrome,[18, 19] and areas of endothelial denudation may promote superficial thrombosis and progression of coronary atherosclerosis.[17, 36, 37] The capacity of HDL to inhibit endothelial cell apoptosis has therefore been suggested as an important potential anti-atherogenic property of HDL.[23-26]

In the present study delipidated $HDL_{Healthy}$ exerted a more profound endothelial anti-apoptotic effect as compared to purified or recombinant apoA1 and reconstituted HDL, compatible with the concept that the HDL proteome is important for the regulation of endothelial anti-apoptotic pathways by HDL. Our HDL proteomics and gene ontology analysis suggested that changes in HDL-bound clusterin and apoC-III in HDL_{CAD} could be relevant for altered effects on apoptosis. Subsequent validation experiments using HDL obtained by different preparative methods provided evidence that a reduced HDL-associated clusterin and an increased HDL-associated apoC-III content contribute importantly to altered effects of HDL_{CAD} on endothelial apoptosis. A previous study by Vaisar et al.[31] on shotgun proteomics analysis of HDL observed a trend for a lower clusterin and a higher apoC-III content in HDL from patients with CAD according to the peptide index analysis, however, no validation experiments were performed. The present study demonstrates for the first time that proteome remodeling in HDL_{CAD} has direct implications on functional properties, i.e. vascular effects of HDL. These findings point to a novel mechanism leading to altered vascular effects of HDL in patients with coronary disease, that at present is of particular interest,

given the disappointing results of several clinical trials examining therapeutic strategies of HDL-cholesterol raising in these patients.

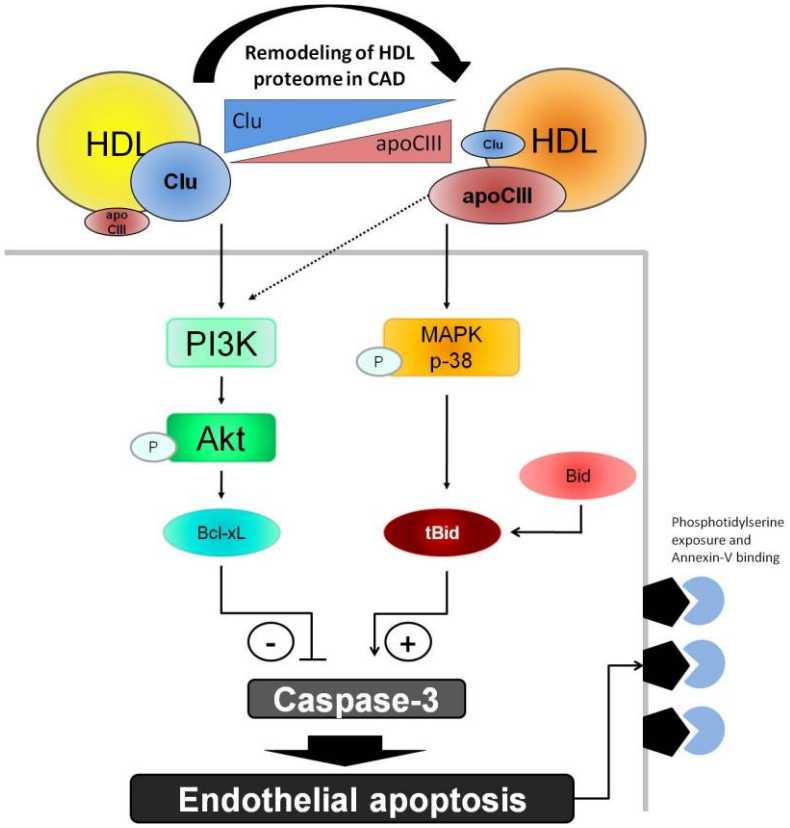

Figure 2.8 Summary of major findings of the study. HDL proteome remodeling in CAD leads to altered effects on endothelial anti-apoptotic and pro-apoptotic pathways. $HDL_{Healthy}$ carries higher amounts of clusterin, that upon induction of apoptosis promotes activation of endothelial PI3K/Akt leading to increased expression of anti-apoptotic Bcl-xL. In CAD, the level of HDL-associated clusterin is reduced, whereas HDL-associated apoC-III content is increased. HDL-associated apoC-III activates MAPK signaling via phosphorylation of p38 leading to increased activation of pro-apoptotic tBid. Our findings therefore suggest that HDL proteome alterations in CAD have implications for the function of HDL with respect to its effects on endothelial integrity and survival.

Clusterin overexpression via adenovirus transfection has been suggested to render endothelial cells more resistant against TNF-α-induced apoptosis.[38] In the present study, however, we have examined the effects of HDL-bound clusterin on endothelial apoptosis using extracellular administration which represents a different setting as compared to overexpression in cultured cells. Notably, addition of clusterin alone did not result in an endothelial anti-apoptotic effect, that was only observed when clusterin was bound to HDL. Clusterin has been suggested to associate with apoA-I[39] and paraoxonase-1.[40] A recent study by Hoofnagle et al.[41] observed that HDL-clusterin levels are reduced in insulin resistant men with a high body-mass-index. It was therefore speculated that a reduced HDL-clusterin-content may have functional implications

for vascular effects of HDL. Of note, a potential anti-atherogenic role of clusterin has been supported by studies examining administration of a clusterin-peptide fragment to apoE-deficient mice and monkeys.[42] In a study of 6 patients with coronary disease it has been observed that combined statin/niacin therapy may promote higher HDL-clusterin-levels in patients with coronary disease.[43] Our present findings provide novel evidence that reduced HDL-clusterin-levels are functionally important with respect to the impaired capacity of HDL_{CAD} to exert endothelial anti-apoptotic effects.

Serum clusterin levels have been reported to be increased in patients with developing coronary heart disease, or myocardial infarction, or type II diabetes[44] and also in response to endotoxin and cytokines[45]. In the present study there was a trend towards increased clusterin-serum-levels. These observations further suggest that changes in the clusterin-content of HDL_{CAD} are specific for HDL, and that the underlying cause is likely a reduced association of clusterin with HDL in patients with CAD rather than systemically reduced clusterin levels.

We observed that HDL-associated clusterin activates via PI3K/Akt the endothelial anti-apoptotic protein Bcl-xL, Bcl-xL belongs to the family of Bcl-2 proteins,[32] has been proposed to be regulated by Akt in tumor cells,[46] and was reported as an important anti-apoptotic protein in endothelial and other cell types.[33] The capacity of $HDL_{Healthy}$ to reduce endothelial apoptosis was not abolished when eNOS was inhibited, although the activation of PI3K/Akt was required for the anti-apoptotic activity of HDL. In line with this observation, a study by Suc et al. has suggested that the protective effect of HDL on endothelial cell survival was not dependent on HDL-associated paraoxonase activity,[25] that we have observed to be critical for the capacity of HDL to stimulate endothelial NO production.[10]

In addition, our study demonstrates that increased levels of apoC-III associated with HDL_{CAD} were responsible for activation of the pro-apoptotic p38-MAPK-signaling pathway in endothelial cells followed by increased expression of the pro-apoptotic protein tBid, a member of Bcl-2 family of proteins.[34, 47] Increased apoC-III levels have been associated with hypertriglyceridemia, metabolic syndrome and diabetes.[48-50] ApoC-III inhibits the clearance of triglyceride-rich lipoproteins.[51] A previous study has shown that LDL containing apoC-III was independently associated with an increased risk of CAD.[52] Furthermore, a recent case-control study has suggested that HDL containing apoC-III is associated with a higher risk of future CHD.[53] Transfers of apoC-III between lipoprotein particles have been previously described.[54] However, in the present study the apoC-III content was not only increased in the HDL fraction but also in the serum and the LDL/VLDL fraction of patients with CAD, suggesting an increased synthesis or a reduced clearance of apoC-III in patients with CAD.

Of note, our findings do not exclude that HDL-associated lipids may also promote endothelial anti-apoptotic effects of HDL. In particular, HDL-associated lysosphingolipids have been suggested to exert

endothelial anti-apoptotic effects,[23] although this has not been observed when sphingosine-1-phosphate (S1P) was added to reconstituted HDL.[26] Interestingly, however, a recent study has examined the S1P content of HDL in patients with coronary disease and an acute coronary syndrome.[55] Notably, in patients within the first hours of an acute coronary syndrome an increased S1P content of HDL was observed, suggesting that it would be unlikely that an altered S1P content could explain the loss of endothelial anti-apoptotic effects of HDL in these patients.[55]

A recent study by Khera et al.[12] supports the notion that HDL function may be more relevant as compared to HDL-cholesterol serum levels alone for development of coronary disease. Khera et al. analyzed the cholesterol efflux capacity of apoB-depleted serum, that was lower in cases with coronary disease as compared to controls, independently of HDL-cholesterol levels. The present study describes for the first time, that effects of HDL on endothelial cell apoptosis are markedly different between $HDL_{Healthy}$ and $HDL_{CAD,}$ that may limit the capacity of HDL to counteract clinical complications of atherosclerosis, since an impaired endothelial integrity has been suggested as an important mechanism promoting clinical complications of coronary disease.

Study limitations. A potential limitation of the present study is the use of cardiovascular drugs in patients with CAD, but not in healthy subjects, that may impact on functional properties and composition of HDL. Previous studies have suggested that statin therapy may reduce apoC-III levels in plasma, HDL or apoB-containing lipoproteins.[56, 57] In the present study most patients were on statin therapy, however, apoC-III levels in LDL/VLDL and HDL were still higher as compared to healthy subjects. While we cannot exclude that medical treatment in addition to the underlying coronary disease may have impacted on altered HDL composition and function in patients with CAD, the present data are consistent with the notion that despite current medical therapy the function and composition of HDL remain abnormal as compared to healthy subjects. Another potential limitation of our study is the use of chloroform/methanol method for delipidation of HDL where we cannot exclude extraction of hydrophobic proteins from HDL. However, since delipidated HDL still exerted a similar effect on endothelial apoptosis as compared to $HDL_{Healthy}$ before lipid extraction (Figure 3B), the anti-apoptotic protein components were likely largely present in the delipidated HDL protein fraction.

In summary, our findings provide novel evidence that remodeling of the HDL proteome in patients with coronary disease has important functional implications with respect to effects of HDL on endothelial cell survival. In particular, we have observed that a reduced clusterin and increased apoC-III content in HDL_{CAD} lead to an impaired effect of HDL on endothelial anti-apoptotic pathways and an activation of pro-apoptotic-signaling in endothelial cells. These findings provide novel insights into mechanisms underlying altered vascular effects of HDL in patients with coronary disease.

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2.7 Supplementary material

2.7.1 Supplementary methods

Patient Characteristics. Patients with stable CAD or an ACS (STEMI or NSTEMI) and healthy subjects (without cardiovascular risk factors) were recruited at the University Hospital of Zurich. The diagnosis of stable CAD or an ACS was made according to the guidelines of the American College of Cardiology/American Heart Association task force.[1, 2] Patients with an ACS (STEMI and NSTEMI) were recruited if they presented within 12hours after the onset of symptoms and were in a fasting state for at least 12hours.

Endothelial cell culture. Human aortic endothelial cells (HAEC) were obtained from Clonetics and cultured in endothelial cell basal medium-2 (Clonetics, USA) supplemented with endothelial growth medium–SingleQuots as indicated by the manufacturer (37°C, 95% air / 5% CO2). SingleQuots contain human epidermal growth factor (hEGF), hydrocortisone, gentamicin and amphotericin-B, fetal bovine serum, vascular endothelial growth factor (VEGF), human fibroblast growth factor-basic (hFGF-B), insulin-like growth factor (R3-IGF-1), and ascorbic acid. HAECS were grown to sub-confluency and rendered quiescent before experiments by incubation in medium containing 0.5% FCS. Endothelial apoptosis induced by serum withdrawal was done by changing the medium to basal medium without supplements. As another stimulus for endothelial apoptosis, cells were exposed to TNF-α (40 ng/ml) for 24 hours as described previously.[3]

Isolation of High-Density Lipoprotein. HDL was isolated as described previously by sequential ultracentrifugation (d = 1.063-1.21 g/ml) using solid potassium bromide (Merck KGaA, Germany) for density adjustment.[4-6] Furthermore, a second method for HDL isolation, i.e. a recently described gel filtration chromatography protocol used for the analysis of the HDL proteome was applied largely as described previously.[7] In brief, 200 microliters serum was applied to two Superdex-200 gel filtration columns (10/300 GL; GE Healthcare, USA) arranged in series on a FPLC system (ÄKTA; GE Healthcare, USA), with a flow rate of 0.3 ml/min in Tris buffer (10 mM Tris, 0.15 mM NaCl, 1 mM EDTA, 0.2% NaN3). The eluate was collected using a fraction collector maintained at 4°C.

HDL-delipidation. For proteomics studies and functional studies HDL was delipidated using methanol/chloroform extraction.[8] In brief, HDL was precipitated by the addition of methanol:chloroform (2:1 vol/vol). Two volumes of water were added and the aqueous upper phase was removed. Then 2 volumes of methanol were added, protein were precipitated and dissolved in 25 mM ammonium bicarbonate at pH 8.0. For proteomics studies of HDL, the samples were reduced and and carboxymethylated prior to digestion by sequencing grade trypsin (Promega, USA) overnight at 37ºC.

Tryptic digests were desalted with a C18 ZipTip (Millipore, USA) before analyzed on a hybrid LTQ-Orbitrap mass spectrometer (ThermoFischer Scientific, USA) interfaced with a nanoelectrospray ion source. For further details of sample preparation and LC-ESI-MS/MS analysis please see the Supplemental Methods. Lipid-free apoA-I was isolated as reported in detail previously.[9]

Preparation of Reconstituted HDL. Reconstituted HDL (rHDL) comprising apoA1, POPC, and cholesterol was prepared with sodium cholate dialysis method[9] using an apoA-I/POPC/cholesterol molar ratio of 1:80:10, as previously reported.[10] Reconstituted HDL containing clusterin or apoC-III was prepared using a similar method with purified clusterin (Prospec, USA) or apoC-III (Academy Biomedicals, USA).

Measurement of Endothelial Cell Apoptosis *In Vitro* using FACS Analysis. Human aortic endothelial cells (HAECs; Clonetics, USA) were collected after detachment with Accutase (PAA Laboratories, Austria) and were resuspended in 140 mM NaCl, 10 mM Hepes, and 2.5 mM $CaCl_2$ and incubated with Annexin V-FITC (Roche Diagnostics, Switzerland) for 30 minutes at room temperature according to the manufacturer's instructions. Flow cytometric analyses were performed using a BD-FACScan flow cytometer (BD Biosciences, USA). Data were analyzed using *FlowJo* software (Treestar. Inc.). For TUNEL staining, endothelial cells were fixed with 4% paraformadehyde, permeabilized with 0.1% Triton X-100 and stained with TUNEL labeling solution (In Situ Cell Death Detection Kit, Fluorescein, Roche, Switzerland) according to the manufacturer's instructions.

Measurement of Endothelial Cell Apoptosis *In Vitro* Using Fluorescence Microscopy. Endothelial cells were grown on glass chamber slides. Following apoptosis induction with or without the presence of HDL, endothelial cells were washed and incubated with Annexin V-FITC (Roche, Switzerland) for 30 minutes at room temperature according to the supplier's instructions. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) suspended in mounting medium (Dako, Baar, Switzerland).and analyzed using a fluorescent microscope (DM-IRB) connected to a digital imaging system (Spot-RT; Diagnostic Instruments/Visitron Systems).

Measurement of Endothelial Cell Apoptosis *In Vitro* Using the Caspase-3 Activity Assay. Caspase-3 activity was measured using a colorimetric assay according to the manufacturer's instructions (CASP-3-C, Sigma, USA). In this assay system, the colorimetric substrate (Ac- DEVD-pNA) is hydrolyzed by caspase-3, releasing p-nitroaniline (pNA) which is monitored by a *Versamax microplate reader (Molecular Devices, USA*) at 405 nm.

Measurement of Endothelial Cell Apoptosis *In Vivo* using FACS Analysis and Active Caspase-3 Staining. Male apoE(-/-).C57BL/6 mice, aged 12-16 weeks, were used for tail-vein injection of HDL (14 mg HDL protein/kg body weight), following anaesthesia with inhalation of isoflurane (3%). Twenty-four

hours after the injection of HDL or PBS-buffer, mice were euthanized and the aorta were harvested and immediately digested for FACS analysis of endothelial cell apoptosis or fixated for histological staining. For flow cytometry analysis of endothelial cell apoptosis, the aorta was digested using 2 mg/ml collagenase buffer at 37 ºC for 45 minutes. Cells were stained with anti-mouse CD31 (BD Pharmingen, USA) and Annexin V-APC (BD Pharmingen, USA). As another approach to verify endothelial cell apoptosis *in vivo* immunofluorescence staining was used. Aortic sections were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with Triton X-100, and blocked with FBS. The anti-mouse CD31 antibody (BD Pharmingen, USA), cleaved caspase-3 antibody (Cell Signaling Technology, USA) and TUNEL In Situ Cell Death Detection Kit, Fluorescein (Roche, Switzerland) were used for staining and the cell nucleus was stained with DAPI. All animal protocols were approved by the local animal care and use committee.

Endothelial cell transfection with eNOS specific small interference RNA. Endothelial cells were transfected with small interfering RNA targeted to eNOS (5´-CCUACAUCUGCAACCACAU-3´) or nontargeting scrambled RNA duplex (5`-GAUCAUACGUGCGAUCAGA-3´) at a final concentration of 15nM using N-TER nanoparticle siRNA transfection system (Sigma-Aldrich, USA) and serum-free cell culture medium according to the manufaturer‘s protocol.

Sample preparations for MS. HDL was precipitated by chloroform:methanol (2:1 vol/vol) extraction followed by centrifugation at 4000 x g for 30 minutes at 4ºC. The protein pellet was dissolved in 25 mM ammonium bicarbonate at pH 8.0. 40 µg of the protein was separated on 1-dimensional SDS PAGE. The protein containing gel lane was cut into 8 pieces. Each individual protein containing gel piece was reduced for 45 minutes at 60ºC in 50 mM ammonium bicarbonate buffer (pH 8.0) containing 20 mM DTT, followed by alkylation of the cysteine residues with the addition of iodoacetamide to a final concentration of 25 mM for 45 minutes at room temperature and darkness. Enzymatic digestion was performed in 25 mM ammonium bicarbonate, pH 8.0 overnight at 37 ºC with 50 ng sequencing grade trypsin (Promega). Peptides were extracted with 80% Acetonitrile / 5% trifluoroacetic acid and dried under vacuum. Tryptic digests were desalted with a C18 ZipTip (Millipore), according to the manufacturer’s instructions, prior to MS analysis.

LC-ESI-MS/MS. Samples were analyzed on a hybrid LTQ-Orbitrap mass spectrometer (ThermoFischer Scientific) interfaced with a nanoelectrospray ion source. Chromatographic separation of peptides was achieved on an Eksigent nano LC system (Eksigent Technologies, Dublin, CA, USA), equipped with a 8 cm in-house made tip column, 75 µm inner diameter, packed with a reverse phase C18 material (AQ, 3 µm 200 Å, Bischoff GmbH, Leonberg, Germany). Peptides were loaded from a cooled (10°C) Spark Holland auto sampler and separated using ACN/water solvent system containing 0.2% formic acid with a flow rate

of 200 nl/min. Peptide mixtures were separated with a gradient from 3 to 40% ACN in 60 minutes. Up to five data-dependent MS2 spectra of the most abundant doubly or triply charged precursor signals were acquired in the linear ion trap using collision induced dissociation. FT-MS spectra were acquired at 60 000 FWHM @ 400 m/z nominal resolution with an overall cycle time of approximately 1.3 second. Dynamic exclusion was switched on, entailing that up to 500 m/z ± 20 ppm values were excluded from tandem MS for 120 second. The automatic gain control was set to 5e5 and 1e4 for full FT-MS and linear ion trap MS2, respectively. The instrument was calibrated externally according to manufacturer's instructions. The Orbitrap calibration was readjusted during data acquisition using internal lock mass calibration on *m*/*z* 429.088735 and 445.120025.

Analysis of MS data. Peak lists were generated using Mascot Distiller software 2.3.2 (Matrix Science Ltd., London, UK). The resulting Mascot Generic Files (mgf) were searched using Mascot server 2.2 [11] against a human protein database downloaded from the European Bioinformatic Institute (EBI, release date: 19/01/2010). The database has been reversed and concatenated to the original database to calculate the false discovery rate. Modifications used for searches included carbamidomethyl (C, fixed, nonhistone searches), phosphorylation (STY, variable), pyro-Glu (N-term Q, variable). Only strictly tryptic peptides [12] with a maximum of 1 missed cleavage site was allowed in database searches. Monoisotopic precursor signals were searched with a tolerance of 6 ppm and an MS/MS signals with a tolerance of 0.5 Da [13, 14].

Relative quantification based on spectral counts. Normalized spectral counts were calculated using Scaffold™ version 2_04_00 (2007 Proteome Software Inc). Database search files generated by Mascot were imported into Scaffold. Peptide and protein identifications were accepted if established at >95.0% probability as specified by the Peptide Prophet [15] and Protein Prophet algorithms [16], respectively. Each identified protein required at least two unique peptides to be part of the data set. To correct for MS/MS sampling differences between individuals, Scaffold outputs were expressed on the basis of the unweighted spectral count assigned to each identified protein. To increase stringency of identification, proteins were required to be present in at least four of the six groups analyzed. The spectral index was used for the relative quantification of each protein of interest.[17] The spectral index was calculated as: $[(S_{CAD}/(S_{CAD}+S_{Healthy})) \times (Np_{CAD}/Nt_{CAD})] - [(S_{Healthy}/(S_{CAD}+S_{Healthy})) \times (Np_{Healthy}/Nt_{Healthy})]$, where S_{CAD} and $S_{Healthy}$ represent unweighted spectral count in $HDL_{Healthy}$ and HDL_{CAD} respectively, Np_{CAD} and $Np_{Healthy}$ represent number of samples where peptides were found for a protein in $HDL_{Healthy}$ and HDL_{CAD} respectively, and Nt_{CAD} and $Nt_{Healthy}$ represent total number of samples of $HDL_{Healthy}$ and HDL_{CAD} respectively. A positive spectral index suggests enrichment of peptides derived from the protein of interest in HDL_{CAD}, whereas a negative spectral index suggests reduction of peptides derived from the protein of interest in HDL_{CAD}.

125I-HDL Binding, Assays. Binding assays were performed as previously described.[18] In brief transfected HEK cells were seeded into 12-well dishes at a concentration of $3x10^5$ cells/well. The assays were performed 48h after seeding in DMEM/25 mM Hepes/0.2% BSA containing 10 μg/ml ^{125}I-HDL, in the absence (total binding) or in the presence (non-specific binding) of a 40-fold excess of unlabelled HDL . After incubation, the amounts of cell associated radioactivity were determined using a Perkin Elmer gamma-counter and the protein content was analyzed as described previously.[18] Specific cell binding were determined by subtracting the values obtained in the presence of the excess unlabelled HDL (nonspecific) from those obtained in the absence of the unlabelled HDL (total).

HEK293 Cell Transfection. The plasmid construct expressing human SR-BI were prepared by cloning human full-length cDNAs into pcDNA 3.1 vector (Invitrogen), and the cDNA sequence was confirmed by DNA sequencing. The HEK293 cells were transfected with the cDNA construct or pcDNA 3.1 vector and clones stably expressing SR-BI, or vector were obtained by G418 selection.

2.7.2 Supplementary results

Role of SR-BI for the endothelial anti-apoptotic effects of HDL

One possible explanation for the anti-apoptotic effect of clusterin could be that it increases binding of HDL to SR-BI. We therefore further investigated the effect of clusterin on the SR-BI-dependent binding of HDL. To examine the specific binding of HDL to SR-BI, we transfected HEK293 cells with SR-BI. There was, however, no significant difference in the SR-BI-specific binding of reconstituted HDL in the presence or absence of clusterin (Supplementary Figure 2.3C), suggesting that the SR-BI mediates, at least in part, the clusterin-dependent endothelial anti-apoptotic effects of HDL, but does not strongly enhance SR-BI-dependent binding of HDL. In contrast, addition of apoC-III to reconstituted HDL did not result in an anti-apoptotic effect that could be blocked by a SR-B1 blocking antibody (Supplementary Figure 2.1C). Furthermore, SR-BI-specific binding of reconstituted HDL was significantly reduced in the presence of apoC-III, that may contribute to the adverse effects of HDL-bound apoC-III on apoptosis (Supplementary Figure 2.3C).

Supplementary Table 2.1 HDL-associated proteins identified in this study.

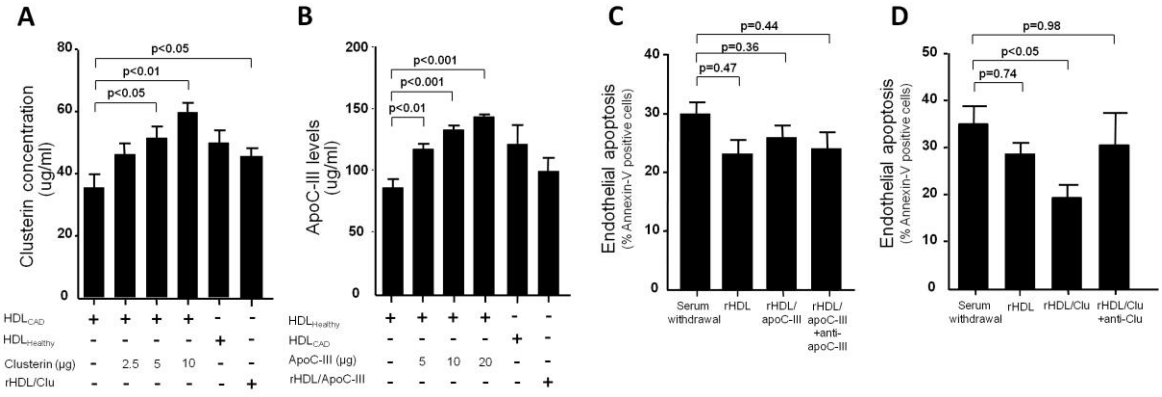
Protein name	Protein short form	Accession number	Spectral index	Median spectral count in $HDL_{Healthy}$	Median spectral count in HDL_{CAD}
Adipocyte plasma membrane-associated protein	APMAP	Q9HDC9-1	0.314	2.5	8.5
Alpha-1-acid glycoprotein 2	ORM2	P19652	0.255	3	2
Alpha-1-antichymotrypsin	SERPINA3	P01011-1	0.255	2	3
Alpha-1-antitrypsin	SERPINA1	P01009-1	-0.069	38.5	28
Alpha-2-antiplasmin	SERPINF2	P08697	0.048	2	3.5
Alpha-2-HS-glycoprotein	AHSG	P02765	0.260	3.5	3.5
Alpha-2-macroglobulin	A2M	P01023	-0.003	1.5	1
Aminopeptidase N	ANPEP	P15144	-0.368	2.5	2
Angiotensinogen	AGT	P01019	-0.166	3	3
Anthrax toxin receptor 1	ANTXR1	Q9H6X2-1	0.149	2	5
Anthrax toxin receptor 2	ANTXR2	P58335-4	0.049	8	12
Antithrombin-III	SERPINC1	P01008	-0.038	2	5
Apolipoprotein A-I	APOA1	P02647	-0.056	512.5	504.5
Apolipoprotein A-II	APOA2	P02652	-0.070	43	45
Apolipoprotein A-IV	APOA4	P06727	0.027	56.5	63.5
Apolipoprotein A-V	APOA5	Q6Q788	0.581	1.5	7.5
Apolipoprotein B-100	APOB	P04114	0.111	350.5	593.5
Apolipoprotein C-I	APOC1	P02654	-0.046	29	25
Apolipoprotein C-II	APOC2	P02655	-0.016	19	15.5
Apolipoprotein C-III	APOC3	P02656	0.197	25	30
Apolipoprotein C-IV	APOC4	P55056	0.173	4.5	8
Apolipoprotein D	APOD	P05090	-0.096	77	77
Apolipoprotein E	APOE	P02649	-0.005	136.5	111.5
Apolipoprotein F (APOF)	APOF	B2RC09	-0.179	7	4.5
Apolipoprotein L1	APOL1	O14791-1	-0.008	55.5	44.5
Apolipoprotein M	APOM	O95445	-0.113	30.5	36
Apolipoprotein(a)	LPA	P08519	0.044	45	49.5
Beta-1A of Integrin beta-1	ITGB1	P05556-1	-0.317	2	2.5
Beta-2-glycoprotein 1	APOH	P02749	0.154	2	4
Beta-3A of Integrin beta-3	ITGB3	P05106-1	-0.534	3.5	2
C4b-binding protein alpha chain	C4BPA	P04003	0.428	5	4
Carbonic anhydrase 6	CA6	P23280	-0.230	4	2
Cathelicidin antimicrobial peptide	CAMP	P49913	-0.085	4	3.5
Cholesteryl ester transfer protein	CETP	P11597-1	0.230	1	3
Clusterin	CLU	P10909-1	-0.323	14	10.5

Complement C3	C3	P01024	0.091	34	62
Complement component 4B (Childo blood group)	C4B	B0UZ85	0.068	11.5	21
Complement component C9	C9	P02748	0.096	5.5	9
Dermcidin	DCD	P81605	-0.106	7.5	8
DPI of Desmoplakin	DSP	P15924-1	-0.068	2.5	2
Endosialin	CD248	Q9HCU0-1	-0.392	2.5	2
GTP-binding protein SAR1a	SAR1A	Q9NR31	0.047	3	4
Haptoglobin	HP	P00738	0.416	2	5
Haptoglobin-related protein	HPR	P00739-1	0.070	4	7.5
Hemoglobin subunit alpha	HBA1	P69905	-0.053	2	1
Hemoglobin subunit beta	HBB	P68871	-0.160	1.5	1
Heparin cofactor 2	SERPIND1	P05546	0.575	1.5	3.5
HLA class I histocompatibility antigen, A-24 alpha chain	HLA-A	P05534	0.083	1.5	2.5
IGH@ protein	IGH@	Q6GMX6	0.601	4.5	3
IGK@ protein	IGK@	Q6PIL8	-0.241	1	1
Inositol monophosphatase 3	IMPAD1	Q9NX62	-0.089	2	1
Integrin alpha-2	ITGA2	P17301	-0.221	2	1
Integrin alpha-IIb	ITGA2B	P08514-1	-0.364	4.5	2
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	Q14624-1	0.042	1	3
Junction plakoglobin	JUP	P14923	-0.260	3	2.5
Lipopolysaccharide-binding protein	LBP	P18428	0.553	2.5	8
Long palate, lung and nasal epithelium carcinoma-associated protein 1	LPLUNC1	Q8TDL5-1	0.277	2.5	7
Multimerin-2	MMRN2	Q9H8L6	-0.322	3	1
Phosphatidylcholine-sterol acyltransferase	LCAT	P04180	-0.192	5.5	4
Phosphatidylinositol-glycan-specific phospholipase D	GPLD1	P80108-1	-0.550	7.5	2
Phospholipid transfer protein	PLTP	P55058-1	-0.142	19.5	20
Plasma protease C1 inhibitor	SERPING1	P05155	-0.170	2.5	1
Platelet-activating factor acetylhydrolase	PLA2G7	Q13093	0.533	1	4
Prenylcysteine oxidase 1	PCYOX1	Q9UHG3	-0.108	23	20.5
Protein AMBP	AMBP	P02760	0.081	1	3
Pulmonary surfactant-associated protein B	SFTPB	P07988	0.784	2	5
Serotransferrin	TF	P02787	0.566	2	3
Serum albumin	ALB	P02768-1	0.024	39	54
Serum amyloid A protein	SAA1	P02735	0.431	3.5	11.5

Serum amyloid A-4 protein	SAA4	P35542	0.007	51.5	61
Serum paraoxonase/arylesterase 1	PON1	P27169	-0.150	48	41
Serum paraoxonase/lactonase 3	PON3	Q15166	-0.121	12.5	11
Sonic hedgehog protein	SHH	Q15465	-0.349	3	1.5
Vesicular integral-membrane protein VIP36	LMAN2	Q12907	0.033	2	3.5
Vitronectin	VTN	P04004	0.281	1	3.5
Hornerin	HRNR	Q86YZ3	0.451	3	4.5

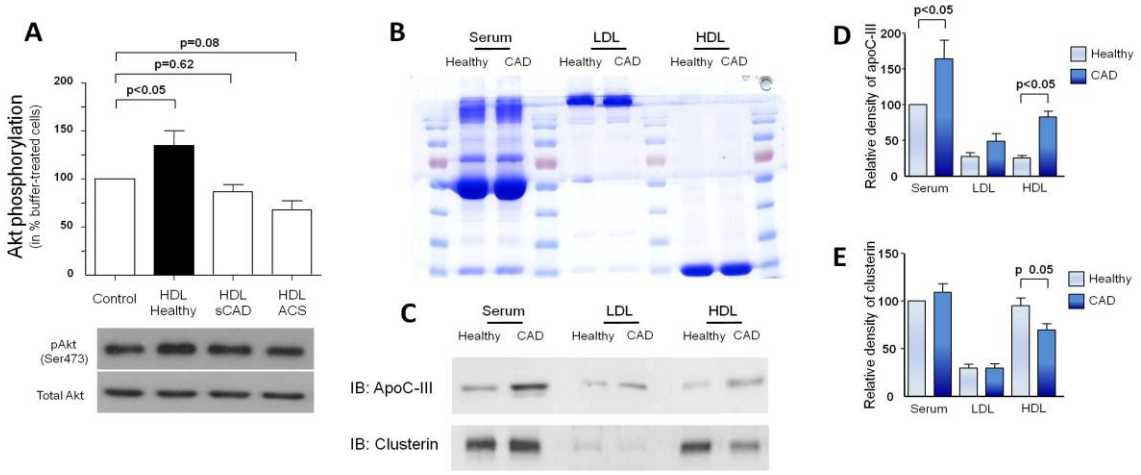
HDL was isolated by sequential ultracentrifugation (d = 1.063-1.21 g/ml) using solid potassium bromide for density adjustment. Isolated HDL samples were reduced and and carboxymethylated prior to digestion by sequencing grade trypsin overnight at 37ºC. Tryptic digests were desalted with a C18 ZipTip before analyzed on a hybrid LTQ-Orbitrap mass spectrometer interfaced with a nanoelectrospray ion source. Database search files generated by Mascot were imported into Scaffold. Peptide and protein identifications were accepted if established at >95.0% probability as specified by the Peptide Prophet and Protein Prophet algorithms. Each identified protein required at least two unique peptides to be part of the data set. To correct for MS/MS sampling differences between individuals, Scaffold outputs were expressed on the basis of the unweighted spectral count assigned to each identified protein. The spectral index was used for the relative quantification of each protein of interest. A positive spectral index suggests enrichment of peptides derived from the protein of interest in HDL_{CAD}, whereas a negative spectral index suggests reduction of peptides derived from the protein of interest in HDL_{CAD}.

Supplementary Figure 2.1



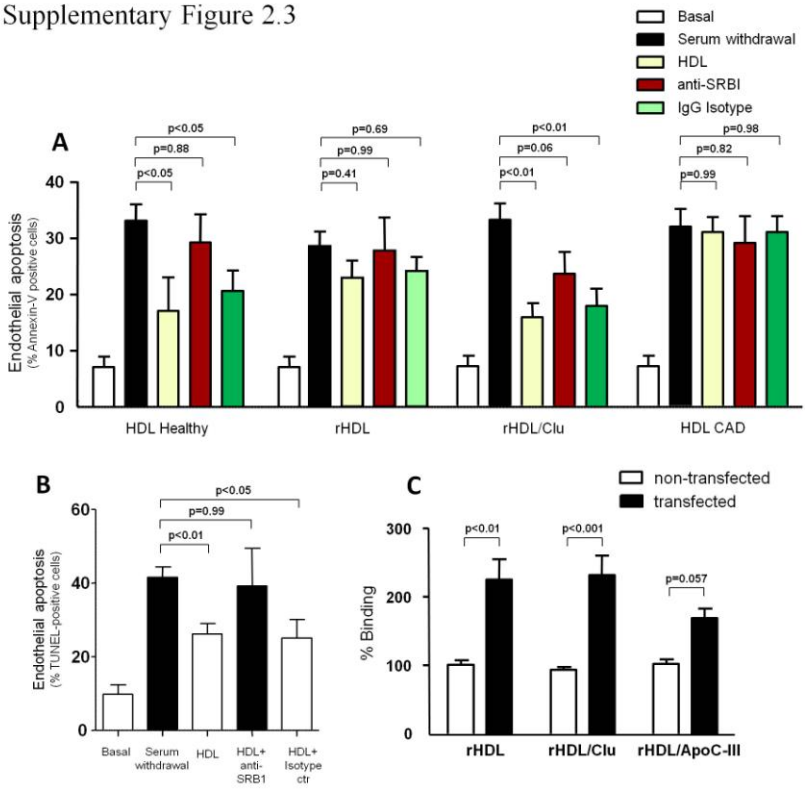
Supplementary Figure 2.1 (A). The amount of clusterin in HDL following reconstitution or addition to $HDL_{Healthy}$. (B) The amount of apoC-III in HDL following reconstitution or addition to $HDL_{Healthy}$ (C) Effect of blocking antibody against apoC-III on the anti-apoptotic capacity of rHDL/apoC-III. (D) Effect of blocking antibody against clusterin on the anti-apoptotic capacity of rHDL/Clu.

Supplementary Figure 2.2



Supplementary Figure 2.2 (A) Effect of $HDL_{Healthy}$, HDL_{sCAD} and HDL_{ACS} **(**50µg/ml) on phosphorylation of Akt at Ser473, normalized to total Akt. (B) Representative coomassie blue-stained SDS-PAGE gel of serum, LDL and HDL from healthy subject and patient with CAD. (C) Western blots analysis of ApoC-III and Clusterin of serum, LDL and HDL from healthy subject and patient with CAD. (D) Densitometric analysis of western blot against ApoC-III. (E) Densitometric analysis of western blot against clusterin.

Supplementary Figure 2.3



Supplementary Figure 2.3 (A) Effect of SR-BI inhibition with specific blocking antibody against SR-BI on the anti-apoptotic capacity of $HDL_{Healthy}$, rHDL, rHDL/Clu and HDL_{CAD} analyzed using FACS analysis with annexin-V staining, and (B) TUNEL staining. (C) Binding of rHDL, rHDL/Clu and rHDL/apoC-III on HEK293 cells transfected with SR-BI.

2.7.3 References to Supplementary Material

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Chapter 3

Endothelial effects of high-density lipoprotein following treatment with CETP inhibitors, dalcetrapib and torcetrapib, in patients with CAD or CAD-risk equivalent

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Manuscript in preparation

Contribution by MR

Design of the study, experiments, data analysis, manuscript writing

3.1 Abstract

As low HDL-Cholesterol levels are associated with an increased cardiovascular risk at least in epidemiology, raising HDL-Cholesterol is being examined as a potentially important therapeutic strategy. Treatment with cholesteryl ester transfer protein (CETP) inhibitors may increase HDL cholesterol levels and are currently being investigated as a potential treatment against cardiovascular disease. However, vascular effects of HDL have recently been found to be highly heterogeneous and benefits may be lost, at least partially, in patients with coronary artery disease (CAD). We therefore studied in patients with CAD or CAD-risk equivalent the effect of the CETP inhibitors, dalcetrapib or torcetrapib, as compared to placebo on HDL function.

HDL was isolated by sequential ultracentrifugation from patients randomized to treatment with dalcetrapib (600 mg/day; n=25) or placebo (n=25) at baseline, 12 and 36 weeks, and from age matched healthy subjects. In parallel, HDL was isolated from patients randomized to receive treatment with torcetrapib (60 mg/day; n=31) or placebo (n=36) at baseline and 6 months. The effect of HDL on endothelial nitric oxide (NO) production was measured by ESR spectroscopy and on endothelial pro-inflammatory and pro-apoptotic activation determined by VCAM-1 and active caspase-3 expression assays, respectively.

There was no significant improvement in the capacity of HDL to stimulate endothelial NO production following dalcetrapib treatment or placebo in patients with CAD or risk equivalents, that remained impaired as compared to HDL-Healthy. HDL isolated from the dalcetrapib group showed an increase in the capacity to reduce endothelial VCAM-1 activation (9.0±6.3% at 12 weeks, 9.7±6.7% at 36 weeks, vs. 5.3±4.5% at baseline; $p<0.05$); but remained significantly impaired (HDL-Healthy: 15.2±6.5%; $p<0.05$). There was also an increase of the capacity of this HDL to reduce active endothelial caspase-3 expression (12.1±8.7% at 36 weeks vs. 6.9±9.9% at baseline; $p<0.05$); however, these endothelial anti-apoptotic capacities remained largely impaired as compared to healthy subjects (18.9±6.6%; $p<0.05$).

No significant improvement was observed in the capacity of HDL to stimulate endothelial NO production, reduce VCAM-1 activation or inhibit active caspase-3 expression following torcetrapib or placebo treatment in patients with CAD. These vascular effects of HDL remained largely impaired as compared to HDL from healthy subjects. Importantly, however, there was no further impairment in the capacity of HDL to stimulate endothelial NO production, reduce VCAM-1 activation or active caspase-3 activation following treatment with torcetrapib.

Despite a significant increase in HDL-C levels (+31% at 36 weeks with dalcetrapib, +63% at 6 months with torcetrapib), treatment with these CETP inhibitors did not restore the potential anti-atherosclerotic properties of HDL on the endothelium as found in the healthy controls. Importantly, these treatments did not further impair the endothelial effects of HDL. This finding may contribute, at least in part, to the lack

of benefit on endothelial function seen in dal-VESSEL and ILLUMINATE trials and demonstrates the importance of measuring not just HDL-C plasma levels but also its vascular impact when assessing novel treatments.

3.2 Introduction

Despite the significant achievement of low-density lipoprotein cholesterol (LDL-C) lowering therapy in reducing the cardiovascular risk, there remains a significant residual cardiovascular risk even after intense statin treatment.[1-4] Low levels of high-density lipoprotein cholesterol (HDL-C) have been identified in epidemiological studies to be inversely correlated to the risk of coronary artery disease.[5] Low HDL-C is an independent predictor of cardiovascular events even in patients intensely treated with statin.[6] HDL-raising therapy is therefore intensely investigated as a potentially important treatment strategy.

The potential anti-atherogenic effects of HDL are attributed not only to its capacity to promote macrophage cholesterol efflux,[7-9] but also to exert endothelial vasoprotective effects, including the capacity to stimulate endothelial nitric oxide production, anti-inflammatory and anti-apoptotic effects.[10, 11] However, recent studies have demonstrated that the protective effects of HDL may be impaired in patients with coronary disease.[12, 13] Importantly, HDL particles isolated from these individuals may rather turn pro-atherogenic.[12]

Cholesteryl ester transfer protein (CETP) is a plasma hydrophobic glycoprotein secreted by the liver that mediates the transfer of cholesteryl ester from HDL to apoB-containing and triglyceride-rich lipoproteins such as LDL and VLDL and reciprocal transfer of triglyceride from from VLDL to LDL and HDL.[14-16] By virtue of the fact that CEs are delivered to the liver by LDL/VLDL for excretion in bile via the indirect pathway, CETP-mediated transfers are part of the RCT pathway and are potentially antiatherogenic.[17] Furthermore, CETP inhibition due to either genetic deficiency or use of inhibitors results in larger HDL-C particles with elevated CE and decreased triglyceride content and a non-HDL fraction with increased triglyceride and decreased CE. These changes lead to an increased HDL-C due to its delayed clearance from plasma.[18]

Treatment strategy that inhibits the activity of CETP leads to increased plasma HDL-C levels. The first CETP inhibitor, torcetrapib, effectively increased HDL-C and lowered LDL-C but was associated with increased mortality,[19] which may be due to off-target effects.[20] Dalcetrapib, a CETP modulator, modestly increased HDL-C without any clinically significant side effects.[21, 22] However, the dal-OUTCOMES trial was stopped recently due to a lack of clinically meaningful efficacy.[23] While the safety studies did not show adverse vascular effects of dalcetrapib on endothelial function, there was no significant improvement on these parameters either.[22, 24]

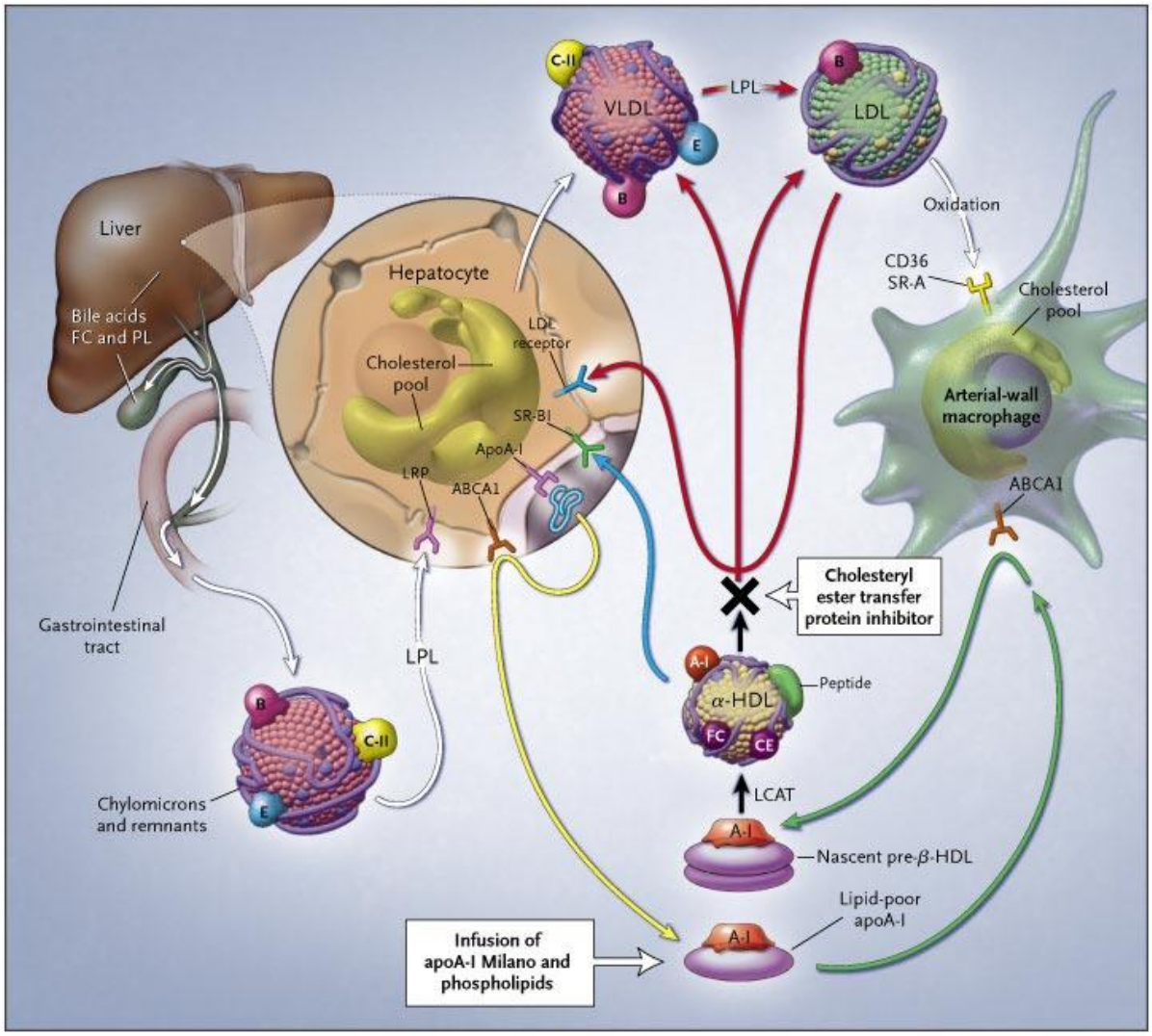


Figure 3.1 HDL cholesterol is returned to the liver through two pathways: selective uptake of cholesterol by the hepatic scavenger receptor, class B, type I (SR-BI, blue arrow), or the transfer of cholesteryl ester by cholesteryl ester transfer protein (CETP) to VLDL–LDL, with uptake by the liver through the LDL receptor (red arrows). FC denotes free cholesterol, PL phospholipids, LRP LDL-related protein, and LPL lipoprotein lipase. Source: Brewer HB Jr., N Engl J Med 2004; 350:1491-1494

These recent failures in treatments that raise HDL-C levels have cast doubt on whether HDL plays a truly protective role in cardiovascular disease. HDL function hypothesis has gained increasing recognition in the field as a better indicator of HDL value in reducing cardiovascular disease risk and it is likely, that only raising of HDL with vasoprotective properties will exert cardiovascular protection. It is therefore essential to develop new metrics for assessing HDL's cardioprotective effects. One diagnostic approach might be the cell-based assays that we recently uncovered to have the potential to distinguish dysfunctional HDL particles in CAD patients.[12, 25]

As discussed in the previous chapter, there may be several mechanisms in play contributing to the altered endothelial protective effects of HDL in CAD. While inactivation of PON-1 may lead to compromised anti-oxidant, anti-inflammatory effects as well as impaired capacity to stimulate NO production,[12] remodeling of the HDL proteome in CAD with respect to clusterin and apoC-III levels may contribute to the activation of pro-apoptotic signaling pathway by HDL.[25] Therefore, our study sought to develop new assays for assessing different HDL functions, in a relatively high-throughput manner. In particular, these assays were developed and optimized to assess the capacity of HDL to stimulate endothelial NO production, promote endothelial anti-inflammatory and anti-apoptotic effects.

Using these assays, we investigated the capacity of dalcetrapib to modulate these endothelial effects of HDL, using HDL isolated from patients with CAD or CAD-risk equivalent treated with CETP inhibitor dalcetrapib or placebo. In addition, using the same study parameters, we sought to investigate the impact of torcetrapib on the endothelial effects of HDL, using HDL isolated from patients with CAD treated with torcetrapib or placebo.

3.3 Cell-based assays for assessment of HDL function

3.3.1 In-cell western assays for assessment of VCAM-1 and active caspase-3 expression

In-cell-western (ICW) technique is a cell-based assay for the measurement of protein expression levels in their cellular context. It is increasingly used to substitute the western immunoblotting technique that is generally labour-intensive, time-consuming and low-throughput. ICW uses near-infrared (NIR) fluorophore–conjugated antibodies which can be analyzed with NIR scanners (Odyssey®) equipped with two separate channels. This enables simultaneous detection of two different proteins, providing that the primary antibodies are raised in different species and that the species-specific secondary antibodies are labeled with different fluorophores. This segregation of signals is particularly useful for normalization of the protein signal to that of the reference protein, e.g. actin, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). ICWs utilize 96-well or 384-well microplates into which adherent or non-adherent cells can be plated and analyzed using the Odyssey® scanner. ICWs eliminate the need for protein harvesting, lysate preparation, electrophoretic separation and electrophoretic transfer steps.

HDL has been shown to inhibit TNF-α stimulated VCAM-1 expression in endothelial cells,[26, 27] and this capacity is lost in patients with CAD.[12] Measurement of VCAM-1 expression upon TNF-stimulation may therefore be relevant for the assessment of the anti-inflammatory property of HDL. Using ICW technique, TNF-α induced endothelial VCAM-1 expression is assessed in the presence or absence of HDL with VCAM-1 primary antibody and near-infrared (NIR) fluorophore–conjugated secondary antibody (Figure

3.2). In addition, measurement is normalized with DRAQ5, near infrared DNA dye. The intra- and inter-assay variabilities are 11.1 and 12.0, respectively.

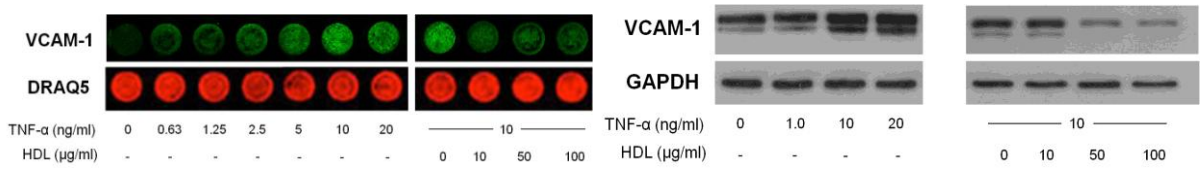


Figure 3.2 Comparison of western blot technique and in-cell western of VCAM-1 expression with increasing amount of TNF-α and different concentrations of HDL.

The capacity of HDL to inhibit endothelial apoptosis has been suggested to be another important anti-atherogenic property of HDL, as demonstrated by inhibition of endothelial caspase-3 activation.[28, 29] Interestingly, the anti-apoptotic effect of HDL has been shown to be independent of eNOS activation and the remodeling of HDL proteome with respect to the clusterin and apoC-III contents were suggested to modulate the endothelial apoptotic signaling pathways by HDL.[25] Measurement of active caspase-3 expression may therefore provide another independent and relevant assessment of HDL function. Using ICW technique, endothelial active caspase-3 expression is assessed in the presence or absence of HDL with active caspase-3 primary antibody and near-infrared (NIR) fluorophore–conjugated secondary antibody (Figure 3.3). In addition, measurement is normalized with DRAQ5, near infrared DNA dye. The intra- and inter-assay variabilities are 13.6 and 15.4, respectively.

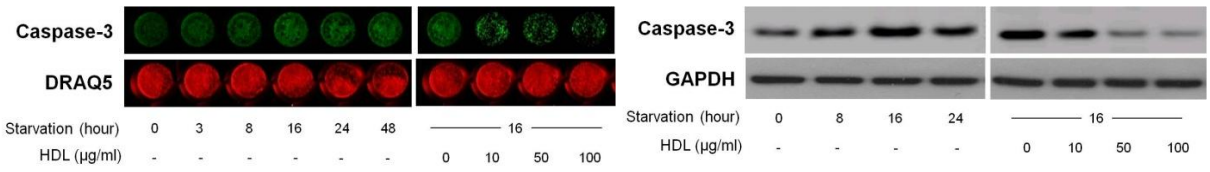


Figure 3.3 Comparison of western blot technique and in-cell western of active caspase-3 expression with different duration of serum starvation and different concentrations of HDL.

3.3.2 Electron Spin Resonance (ESR) spectroscopy for the assessment of endothelial NO bioavailability

Endothelial NO bioavailability was determined by ESR spectroscopy using the spin-probe colloid Fe(DETC)2 (Noxygen), after stimulation with HDL, as previously described.[12] The formed nitrosyl iron(II) complex, NO-Fe(DETC)$_2$, exhibits a characteristic signal with three peaks (Figure 3.4). Signals

were quantified by measuring the total amplitude after correction of baseline. The amount of NO was determined by measuring the total amplitude of the ESR signal for each treatment.

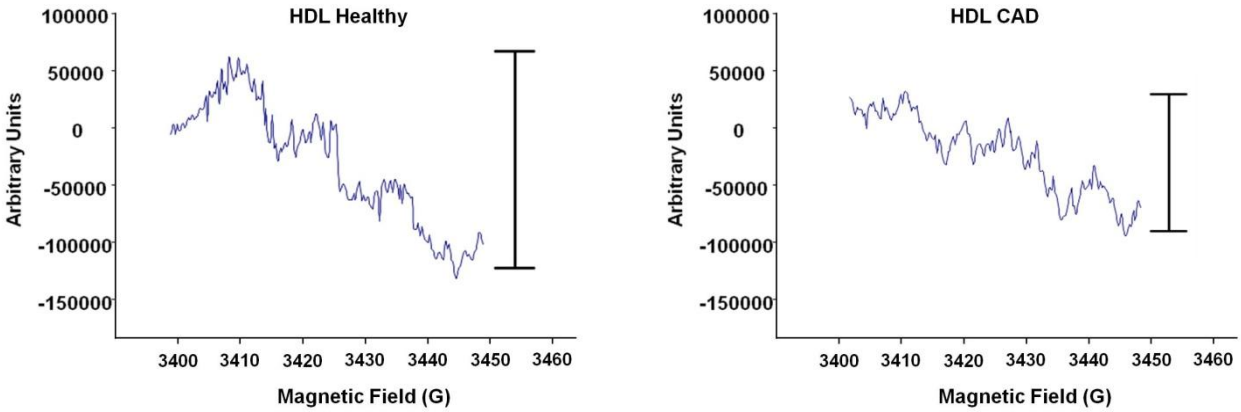


Figure 3.4 After one hour of stimulation with 50μg/ml HDL, which was isolated from healthy subjects, there was a significant increase in NO production. Following stimulation with HDL from patients with stable CAD there was no increase in NO bioavailability. (Source: Besler et al. J Clin Invest 2011; 121:2693-2708)

3.4 Endothelial effects of HDL remain impaired following dalcetrapib treatment in patients with coronary heart disease (CHD) or risk equivalent

HDL was isolated from 50 patients with CHD or CHD risk equivalent treated with either placebo or dalcetrapib 600 mg/day at baseline level, 12 weeks and 36 weeks. The characteristics of the study population are shown in Table 3.1.

3.4.1 Effects of dalcetrapib on HDL capacity to stimulate endothelial nitric oxide (NO) production and to promote anti-inflammatory and anti-apoptotic functions

HDL isolated from patients with CAD or risk equivalent treated with dalcetrapib or placebo showed no significant capacity to stimulated endothelial NO production. In contrast, HDL from healthy subjects markedly stimulate endothelial NO production as measured by ESR spectroscopy. HDL isolated from patients treated with dalcetrapib showed an increased capacity to inhibit endothelial VCAM-1 activation (9.0±6.3% at 12 weeks, 9.7±6.7% at 36 weeks, vs. 5.3±4.5% at baseline; $p<0.05$).

Table 3.1 Baseline demographics and clinical characteristics

Parameter	Placebo (N=25)	Dalcetrapib (N=25)

Age, years	61.3 (1.6)	61.9 (1.4)
Male sex, n (%)	25 (100)	25 (100)
BMI, kg/m^2	28.8 (0.8)	28.8 (0.8)
Systolic blood pressure 24 hour mean	120.8 (2.5)	130.9 (2.0)
Diastolic blood pressure 24 hour mean	73.4 (1.4)	77.3 (1.7)
Total cholesterol, mg/dL	144.5 (3.7)	156.6 (6.0)
HDL-C, mg/dL	38.2 (1.4)	38.4 (1.4)
LDL-C, mg/dL	80.0 (2.2)	85.7 (5.0)
Triglycerides, mg/dL	131.6 (12.1)	161.9 (15.6)
hsCRP, mg/L	2.5 (0.7)	4.2 (1.8)

Data are mean (SE), unless otherwise stated. BMI, body mass index; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; hsCRP, high sensitivity C-reactive protein.

Table 3.2 Percent change from baseline in HDL-C level post-treatment

Treatment Arm	**Mean (SD)**	**Median (Q1, Q3)**	**Min**	**Max**
Dalcetrapib, week 12	30.48 (14.42)	28.57 (19.35, 38.46)	9.52	71.43
Dalcetrapib, week 36	43.62 (20.04)	39.39 (30.95, 54.29)	4.17	103.23
Placebo, week 12	-0.25 (11.81)	0 (-6.38, 3.33)	-28.95	29.03
Placebo, week 36	0.14 (11.99)	0 (-7.89, 6.90)	-21.05	31.25

However, the effect is significantly lower as compared to the capacity of HDL from healthy subjects to reduce endothelial VCAM-1 expression (HDL-Healthy: 15.2±6.5%; p<0.05). HDL isolated from patients treated with dalcetrapib showed an increased capacity to reduce active endothelial caspase-3 expression (12.1±8.7% at 36 weeks vs. 6.9±9.9% at baseline; p<0.05); however, these endothelial anti-apoptotic capacities remained largely impaired as compared to healthy subjects (18.9±6.6%; p<0.05) (Figure 3.5). Furthermore, there was no statistical difference between the dalcetrapib and placebo groups for the change from baseline in the capacity of HDL to stimulate endothelial NO production, to reduce VCAM-1 expression or to inhibit caspase-3 expression (Figure 3.5).

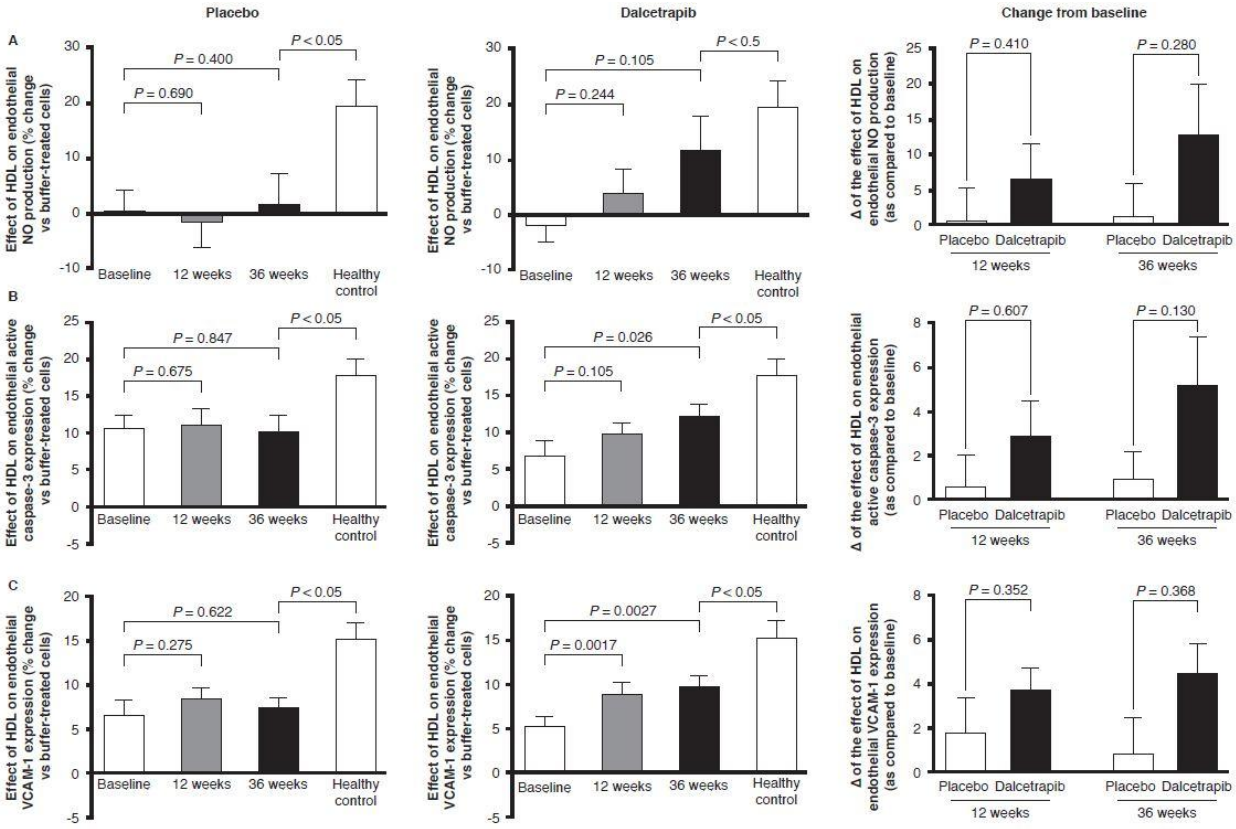


Figure 3.5 Effects of dalcetrapib on HDL capacity to stimulate endothelial NO production (A), endothelial anti-apoptotic activity (B), and endothelial anti-inflammatory activity (C).

3.5 Impact of torcetrapib treatment on HDL effects on endothelial cells

HDL was isolated from 50 patients with CHD treated with either placebo or torcetrapib 600 mg/day at baseline level, 12 weeks and 36 weeks. The characteristics of the study population are shown in Table 3.3. Change from baseline in HDL cholesterol levels following treatment is 63.1±28.7% in the torcetrapib treatment arm group, in comparison to 1.7±12.7% in the placebo treatment arm group.

Table 3.3 Baseline demographics and clinical characteristics

Parameter	Placebo (N=30)	Torcetrapib (N=42)
Age, years	63.2 (7.5)	65.6 (7.4)
Male sex, n (%)	38.9 (100)	52.8 (100)
BMI, kg/m^2	33.1 (7.1)	29.9 (3.4)
Systolic blood pressure 24 hour mean	122.9 (8.6)	122.9 (11.0)
Diastolic blood pressure 24 hour mean	72.8 (7.3)	72.1 (9.3)
HDL-C, mg/dL	40.3 (11.3)	43.6 (8.6)
LDL-C, mg/dL	79.3 (25.0)	72.1 (20.0)
Triglycerides, mg/dL	182.0 (99.6)	159.6 (75.1)
hsCRP, mg/L	2.7 (2.0)	4.1 (11.0)

Data are mean (SD), unless otherwise stated. BMI, body mass index; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; hsCRP, high sensitivity C-reactive protein.

3.5.1 Effects of torcetrapib on HDL capacity to stimulate endothelial nitric oxide (NO) production and to promote anti-inflammatory and anti-apoptotic functions.

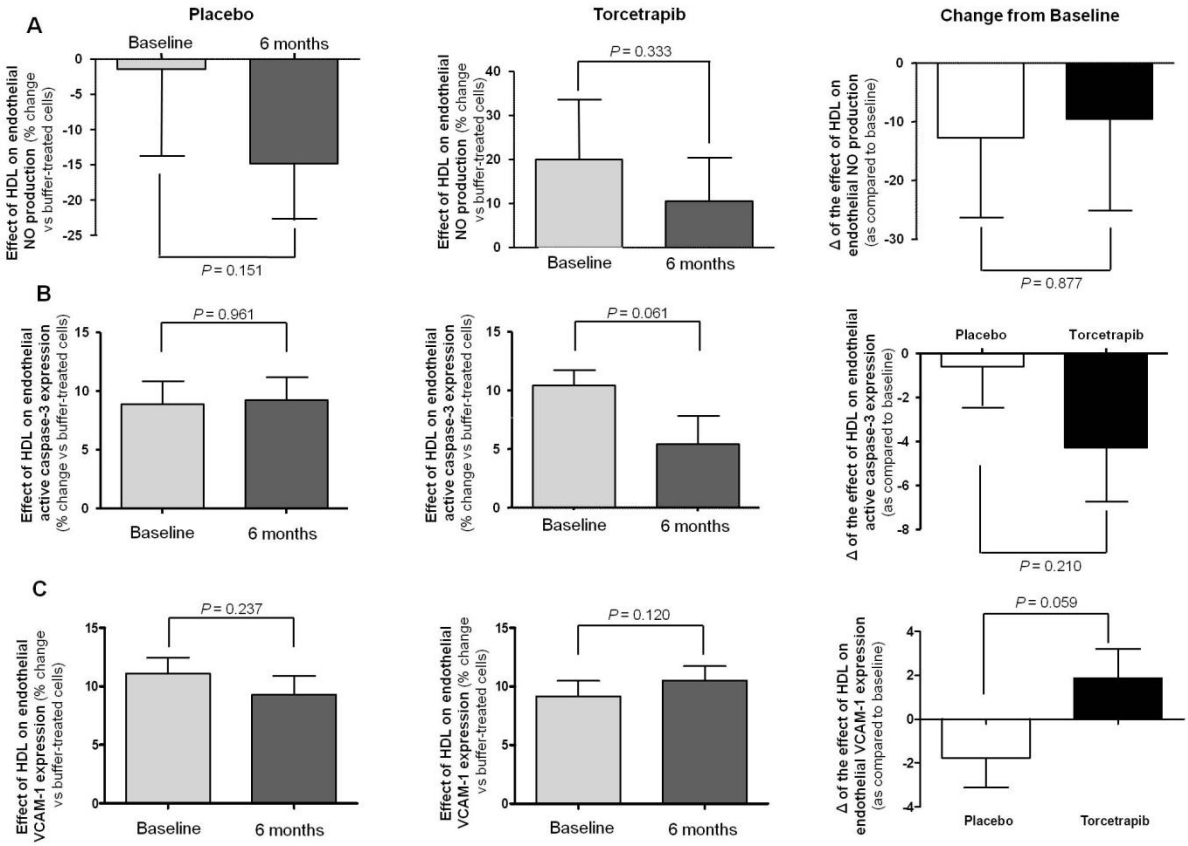
There was no significant improvement in the capacity of HDL to stimulate endothelial NO production, reduce VCAM-1 activation or inhibit active caspase-3 expression following torcetrapib or placebo treatment in patients with CAD (Figure 3.6). These vascular effects of HDL remained largely impaired as compared to HDL from healthy subjects. Importantly, however, there was no further impairment in the capacity of HDL to stimulate endothelial NO production, reduce VCAM-1 activation or active caspase-3 activation following treatment with torcetrapib.

3.6 Discussion

In this study, we have optimized and used cell-based assays for the assessment of HDL effects on the endothelium following treatment with CETP inhibitors, dalcetrapib and torcetrapib. We observed no statistical difference between the dalcetrapib and placebo treatment arm groups for the change from baseline in all HDL functions measured. Interestingly, HDL isolated from patients following treatment

with dalcetrapib showed an increased in the capacity to inhibit VCAM-1 activation and to reduce active caspase-3 expression. However, these effects were still very much impaired as compared to HDL isolated from age matched healthy subjects. On the other hand, there was also no significant improvement in the HDL functions measured following torcetrapib or placebo treatment in patients with CAD. Notably, no impairment in HDL functions was observed following torcetrapib treatment.

Figure 3.6. Effects of torcetrapib on HDL capacity to stimulate endothelial NO production (A), endothelial anti-apoptotic activity (B), and endothelial anti-inflammatory activity (C).



Treatment with CETP inhibitors raises HDL-C levels, which has been proposed to reduce cardiovascular disease risk. However, despite significant elevations in the HDL-C levels, results from recent trials have not shown the clinical benefits of torcetrapib or dalcetrapib. Treatment with torcetrapib, the first CETP inhibitor, increased cardiovascular mortality,[19] which may be due to off-target effects.[20] Meanwhile, clinical trial assessing dalcetrapib treatment was terminated due to a lack of clinically meaningful efficacy.[23]

These observations likely suggest that measurement of HDL-C level is not sufficient to reflect the cardioprotective capacity of HDL and other functional readouts may be better indicators for assessing the cardiovascular risk in an individual. However, given the heterogeneity of HDL particle compositions, different components of HDL may account for different biological functions. As described in Chapter 1, the anti-inflammatory capacity of HDL has largely been attributed to apolipoprotein A-I[30-33] and impairment in the anti-inflammatory effects of HDL have been proposed to be due to displacement by serum amyloid A (SAA)[34-38] as well as oxidative modifications of the apolipoprotein.[39-42] On the other hand, we have observed that HDL-associated antioxidant enzyme paraoxonase-1 (PON1) is an important mediator of the capacity of HDL to stimulate endothelial NO production.[12] In contrast, lipid-free apoA-I did not stimulate eNOS.[9] Other components of HDL, i.e. clusterin and apolipoprotein C-III, have been shown to contribute to the effects of HDL on endothelial apoptotic signaling processes.[25] Measurements of these endothelial effects may therefore provide functional assessment of the different particles or subpopulations that contribute to distinct HDL functions.

Interestingly, despite improvement in the capacity of HDL to inhibit VCAM-1 activation and to reduce caspase-3 expression following dalcetrapib treatment, these effects were still largely impaired when compared to healthy subjects. Treatment with dalcetrapib failed to restore HDL functions to the level of healthy subjects. Importantly, there was no difference when we compared changes from baseline between placebo and dalcetrapib treatment groups in all the HDL functional readouts. Our observations may explain, at least in part, the lack of benefit on endothelial function seen in the dal-VESSEL trial.[24]

Interestingly, in a phase IIb study, dalcetrapib treatment was shown to increase apoA-I level and improve HDL-induced cholesterol efflux mediated by ABCA1 and SR-B1, as compared to placebo-treated group.[43] However, the study did not include comparison with HDL isolated from healthy subjects. It is therefore difficult to evaluate whether dalcetrapib treatment fully restore the cholesterol efflux capacity of HDL. Another possible explanation is that the measurement parameters in the study do not reflect clinically relevant HDL function for the assessment of cardiovascular risk.

We observed no significant improvement in the HDL functions measured following torcetrapib or placebo treatment in patients with CAD. Nonetheless, no impairment in HDL functions was observed following torcetrapib treatment. These findings may contribute to understanding the reason for the failure of torcetrapib. One of the main questions in the failure of torcetrapib is whether the pronounced elevations in the HDL-C levels may give rise to HDL particles that are pro-inflammatory. In this study, we found that while no improvement in endothelial functions afforded by HDL was observed, no further impairment in these parameters were seen either. While our findings support the lack of improvement in the quality of HDL following torcetrapib treatment, the adverse effects observed in the clinical trial of torcetrapib were

likely caused by off-target side effects, which may have been due to direct deleterious actions of torcetrapib on endothelial function,[44] independent of the HDL-cholesterol raising effect.

3.7 Conclusion

HDL cholesterol level is not an ideal marker to capture the cardioprotective potential of HDL. Accumulating evidence has shown that HDL functionality may better predictor of cardiovascular risk. Measurements of specific functional readouts such as the anti-inflammatory, anti-apoptotic and the NO-stimulating capacity may provide better assessment of the quality of HDL particles. Importantly, there is still a need to further understand which functional readouts are most relevant to cardiovascular protection, since it is likely that raising HDL with atheroprotective properties may have beneficial effects on reducing cardiovascular risk. Treatment with CETP inhibitors dalcetrapib and torcetrapib did not restore the potential anti-atherosclerotic properties of HDL on the endothelium as found in the healthy controls, which may contribute, at least in part, to the lack of benefit on endothelial function seen in dal-VESSEL and ILLUMINATE trials. Our findings highlight the importance of measuring not just HDL-C plasma levels but also its vascular impact when assessing novel treatments

3.8 Methods

Isolation of High-density lipoprotein. HDL was isolated as described previously by sequential ultracentrifugation (*d* = 1.063-1.21 g/ml) using solid potassium bromide (Merck KGaA, Germany) for density adjustment.[12, 45, 46]

Measurement of endothelial nitric oxide (NO) production by electron spin resonance spectroscopy. The effects of HDL (50 μg/ml; 60 min, 37°C) on endothelial NO production (HAECs; passage 4-7; Clonetics) was examined by electron spin resonance (ESR) spectroscopy using the spin-probe colloid Fe(DETC)2 (Noxygen), as described and validated previously.[45, 47-49] In brief, ESR spectra of samples frozen in liquid nitrogen were recorded on a Bruker e-scan spectrometer (Bruker BioSpin) with the following instrumental settings: center field (B0) 3425 G, sweep width 80 G, microwave power 39.72 db, amplitude modulation 10.34 G, sweep time 10.49 sec, number of scans 10.

Measurement of endothelial VCAM-1 expression. Endothelial cells were seeded in 96-well plates ($2x10^4$ cells per well) and used for experiments upon reaching confluency. When indicated, cells were pre-incubated with HDL (50ug/ml) overnight at 37°C. Endothelial VCAM-1 expression was induced by addition of TNF-α (10ng/ml) for 4 hours at 37°C. In-Cell Western assays were performed using a In-Cell Western Kit (LI-COR Biosciences, Lincoln, NE). Cells were fixed using 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with Odyssey Blocking Buffer, and incubated with goat anti-human VCAM-1 antibody (R&D Biosciences) at a dilution of 1: 1000 overnight at 4°C. The secondary antibody for goat IgG, which were labeled with IRDye 800CW were subsequently added at a dilution of 1:1,000 in the dark. DRAQ5 was used for normalization. The cells were imaged using a LI-COR Scanner and analyzed by the Odyssey System (Li-COR Biosciences).

Measurement of endothelial active caspase-3 expression. Endothelial cells were seeded in 96-well plates ($2x10^4$ cells per well) and used for experiments upon reaching confluency. Endothelial apoptosis was induced by serum and growth factor deprivation for 20hours at 37°C. When indicated, cells were co-incubated with HDL (50ug/ml). In-Cell Western assays were performed using a In-Cell Western Kit (LI-COR Biosciences, Lincoln, NE). Cells were fixed using 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with Odyssey Blocking Buffer, and incubated with mouse anti-human active caspase-3 antibody (Abcam) at a dilution of 1: 1000 overnight at 4°C. The secondary antibody for goat IgG, which were labeled with IRDye 800CW were subsequently added at a dilution of 1:1,000 in the dark. DRAQ5 was used for normalization. The cells were imaged using a LI-COR Scanner and analyzed by the Odyssey System (Li-COR Biosciences).

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Chapter 4

HDL proteomics analysis with Selected Reaction Monitoring: Relation to clinical outcome

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Contribution by MR

Design of the study, experiments, data analysis

4.1 Abstract

Epidemiological evidence suggests that a higher HDL-C level is associated with a lower cardiovascular disease risk in general populations. Raising HDL is therefore being examined as a potential therapeutic strategy. However, vascular effects of HDL appear to be highly heterogenous and benefits of HDL function may be lost, at least partially, in patients with coronary artery disease (CAD). Recent proteomics studies suggest that HDL proteins that are associated with diverse biological functions. In CAD patients, HDL protein compositions are altered which may have adverse effects on the atheroprotective properties of HDL.

In this study, we used quantitative proteomics to evaluate changes in the HDL proteome and its relation to cardiovascular outcome. HDL was isolated from patients with coronary artery disease derived from the Ludwigshafen Risk and Cardiovascular Health (LURIC) study who died from cardiovascular events versus those who did not develop any events during the entire follow-up study period. HDL-associated proteins were analyzed by a selected reaction monitoring (SRM) experiment in a tandem mass spectrometer resulting in the direct detection and quantification of both the native peptide and isotope labeled AQUA internal standard peptide.

Statistical analysis using SRMStats identified statistically significant differences in the protein compositions of HDL between CAD patients who had a major cardiovascular event versus patients without any cardiovascular event. Alterations in the protein composition of HDL may provide insight into the adverse changes in the vascular effects of HDL and potentially identify new mechanisms of action of HDL, which may be important in the development of novel HDL-targeted therapy.

4.2 Introduction

As highlighted in previous sections, alterations in HDL function are likely, at least in part, attributed to changes in the compositions of HDL particles. The HDL class of particles is structurally and metabolically heterogeneous, due its diverse metabolic origin and by continuous remodeling of the particles by lipolytic enzymes, lipid transporters, and furthermore by lipid and apolipoprotein exchange with other circulating lipoproteins and tissues.[1, 2]

Early study has identified that HDL isolated during the acute phase response have profound changes in its associated proteins and apolipoprotein composition.[3] This HDL is enriched in C-reactive protein (CRP), secretory phospholipase A_2-IIa ($sPLA_2$-IIa), serum amyloid A (SAA), and cholesterol ester transfer protein (CETP). While such remodeling has been suggested to preserve the acceptor role of HDL particles in cholesterol efflux from cells,[4] it may also compromise other functions of HDL. HDL carrying oxidatively modified apolipoprotein A-I (apoA-I) has also been shown to lose its anti-inflammatory effects.[5] Recently, we observed that reduced clusterin and increase apolipoprotein C-III (apoC-III) contents in HDL isolated from patients with CAD may lead to activation of pro-apoptotic signaling in endothelial cells.[6] These observations suggest that a more complete picture of the relationship between HDL functions in physiological and pathological conditions and changes in its protein compositions may be obtained by unbiased evaluation of the HDL-associated proteins. Such knowledge will also be important in identifying novel functions of HDL.

Various functions of HDL have been identified that include participation in lipid metabolism, atheroprotective effects, and also in the innate immune response. Early HDL proteomic studies aimed to identify proteins that could provide clues about the diverse postulated function of this lipoprotein. On the other hand, more recent studies have compared the lipoprotein proteome of healthy controls with that from patients suffering atherosclerotic cardiovascular disease or other metabolic alterations in which changes in HDL levels or subclasses distribution have been previously documented.

Application of quantitative proteomics will allow for the identification of targets for novel HDL-based therapies and the discovery of biomarkers which can be used for diagnostics, prevention and monitoring of cardiovascular disease risk. Importantly, quantitative assessment of HDL proteome will enable a greater understanding of the mechanisms of action of HDL and its alterations in cardiovascular diseases.

4.2.1 Proteomic platforms used for evaluation of the HDL proteome

Qualitative and quantitative analyses of HDL proteins have been reported with the use of 1-D/ 2-DE in combination with proteolytic fragmentation, peptide separation by high performance liquid chromatography (HPLC), and protein identification with double MS in tandem (MS/MS) or with matrix-

assisted laser desorption/ionization–time flight mass spectrometry (MALDI-TOF-MS).[7, 8] Proteomics analysis with 2-DE has several limitations including reproducibility issue and laborious time-consuming processes. Furthermore, considerable loss of materials is often an issue during protein fractionation procedure.

Recent studies have used direct evaluation of protein digests separated by 2D-LC or directly analyzed by MS/MS. Shotgun proteomics has been applied to analyze HDL protein compositions.[6, 8-10] It should be noted, however, that quantification of individual proteins or peptides from complex mixtures or proteolytic digests by MS/MS is still difficult because the most abundant components of proteins/peptides mixtures limit ionization of the less abundant ones. In addition, as pointed out by Duncan et al,[11] in complex proteomes several proteins can be precursors of the same peptide and as a result, depending on the peptide(s) selected, the precursor protein levels may be significantly under- or overestimated.

More recently, SELDI TOF-MS, or *ProteinChip* technology, has emerged to allow for more convenient comparative analysis of the proteome associated with specific metabolic alterations or pathologies with those of lipoproteins from healthy individuals. SELDI was used to analyze LDL[12] or HDL[13-16] that were directly adsorbed onto the SELDI plate. This method allows handling of several individual analyses per week and requires minute amounts of the protein mixtures. However, the limitation of this technique is that protein identities are not revealed in the process because the analytes cannot be subjected to digestion or tandem mass spectrometry. It requires downstream analysis, digestion, and identification of analytes after the quantification step, and its advantages and limitations have been recently reviewed.[17] Furthermore, identification in SELDI is based on low mass resolution data, thereby limiting its application.

Most of the techniques used in the analysis of the HDL proteome currently available in the literature have so far been semi quantitative analyses to compare changes in the lipoprotein proteomes associated with specific metabolic alterations or pathologies with those of lipoproteins from healthy individuals, and often with low number of samples due to highly laborious processes. Meanwhile, recent advances in the mass spectrometry field has allowed for more quantitative analysis or even absolute quantification of proteins in highly complex mixture, with the use of targeted proteomics using selected reaction monitoring (SRM).[18, 19] The main advantage of SRM is the capacity for faster and cost-efficient assay development.[18] SRM has also the feature of being able to quantify multiple proteins in parallel (multiplexing) at a low limit of detection (LOD) and high accuracy. Furthermore, study has shown that protein quantification by SRM in complex samples using predefined assay coordinates is reproducible across different laboratories and instrument platforms.[20]

Targeted mass spectrometry through selected reaction monitoring (SRM) exploits the unique capabilities of triple quadrupole (QQQ) MS for quantitative analysis. In SRM, the first and the third quadrupoles act as

filters to specifically select predefined *m*/*z* values corresponding to the peptide ion and a specific fragment ion of the peptide, whereas the second quadrupole serves as collision cell. Several such transitions (precursor/fragment ion pairs) are monitored over time, yielding a set of chromatographic traces with the retention time and signal intensity for a specific transition as coordinates. The two levels of mass selection with narrow mass windows result in a high selectivity, as co-eluting background ions are filtered out very effectively. Unlike in other MS-based proteomic techniques, no full mass spectra are recorded in QQQ-based SRM analysis. The non-scanning nature of this mode of operation translates into an increased sensitivity by one or two orders of magnitude compared with conventional 'full scan' techniques. In addition, it results in a linear response over a wide dynamic range up to five orders of magnitude. This enables the detection of low-abundance proteins in highly complex mixtures, which is crucial for systematic quantitative studies.

4.2.2 Recent advances in the analysis of HDL Proteome and its pathological remodeling in cardiovascular disease

Using 2-DE in combination with MALDI-TOF-MS, HDL was found to contain besides the classical apolipoproteins: apoAI (6 isoforms), apoAII, apoAIV (6 isoforms), apoE (6 isoforms), apoM (2 isoforms), apoCII, apoCIII (3 isoforms), other proteins as SAA (2 isoforms), α-amylase, and α-1-antitrypsin. Interestingly, although the methods used in these ground-breaking studies are considered semiquantitative, the authors already reported differences in the distribution of apolipoprotein and associated proteins between the large HDL2 and the small HDL3 subclasses that have been confirmed by more recent studies.[21]

It has been suggested that proteins associated with HDL could be separated into clusters whose members shared participation in defined functional areas. These areas were: lipid metabolism and transport, inflammatory markers, immune system and complement factors, growth factors, hormone-binding proteins, and proteins involved in hemostasis.[8, 22] Recent data from Davidson et al. supports the hypothesis that proteomics of HDL subpopulations may lead to a more comprehensive understanding of differential functions of individual particle subclasses.[23] In these experiments HDL2b,- 2a, -3a, -3b, and HDL3c were isolated by isopycnic density gradient ultracentrifugation in neutral salts, and 28 different HDL-associated proteins and apolipoproteins were identified in each subclass using LC/ESI/MS and a quadropole TOF analyzer. The identified proteins appear to associate into 5 distinct clusters with different abundance of the individual proteins in the HDL subclasses. Furthermore, there are strong correlations between levels of the components in the clusters of individual subclasses and their potential antioxidant properties. The authors proposed that this is specially the case for the dense HDL3 subclass that shows elevated content of the cluster containing PON 1/2, PON 3, and apoL-I. Moreover this dense HDL3 subclass appears to be the

unique carrier of apoA-IV, PON1, phospholipid transport protein (PLTP), apoJ, apoF, and apoL-I. In the other hand, the apparent preference of apoE, apoCII and apoCIII for the light HDL2 subclass was proposed to be related to the known interaction of these particles with components of the LDL receptor family.[23]

Given the complexity of HDL and its multitude of biological functions, it is conceivable to expect that sets of functionally associated proteins can provide information about their participation in the spectrum of atheroprotective actions attributed to HDL. This has further led to studies in which the aim became to compare the HDL proteome of healthy subjects with that of particles from patients with dyslipidemias or CAD.[6-9] Some of the studies aim to document differences that may allow the assignment of specific proteins to functional defects of HDL.

In the study by Vaisar et al., HDL was shown to carry apolipoproteins and proteins with functions in lipid metabolism, the acute phase response, complement regulation, and blood coagulation.[8] In a recent study from the same laboratory the proteome of HDL3 from 6 CAD patients was compared before and after 1-year treatment with combined statin/niacin therapy.[9] In this study, HDL3 of CAD patients is significantly enriched in apoE and apoCII and contain less apoJ and phospholipid transport protein (PLTP) than the particles from controls. Niacin/statin treatment of the patients decreases HDL3 apoE to the levels of healthy controls.[9] The authors proposed that an increase in apoE could accelerate hepatic removal of apoE-rich HDL3 and that this is responsible for its low plasma level in patients with CAD. Thus, decrease in apoE-rich HDL removal, caused by the niacin/statin treatment, may lead to the increase number of circulating particles. The treatment, on the other hand, raises apoJ and PLTP in HDL3; a change that was suggested may improve the participation of HDL3 in reverse cholesterol transport (RCT).[9]

In our recent study (please refer to Chapter 2), we have observed that reduced clusterin and increased apolipoprotein C-III content in HDL isolated from patients with CAD lead to activation of pro-apoptotic signaling pathways in endothelial cells.[6] In contrast to HDL from healthy subjects, HDL isolated from patients with stable CAD or an acute coronary syndrome failed to inhibit endothelial cell apoptosis *in vitro* and in apoE-deficient-mice *in vivo*. Instead, HDL isolated from these patients stimulated endothelial pro-apoptotic pathways, in particular p38-MAPK-mediated activation of the pro-apoptotic Bcl-2-protein tBid. Our studies further suggest that differences in the proteome of HDL from patients with CAD, in particular reduced HDL-associated clusterin and increased HDL-associated apoC-III, play an important role for altered activation of endothelial anti- and pro-apoptotic signaling pathways.[6]

Taken together, these studies indicate that the HDL proteome can change in a variety of disease states and these changes are often related to at least *in vitro* measures of HDL function. However, it remains to be

seen whether these changes are secondary to other processes occurring during disease progression or if the HDL particles themselves contribute to the disease etiology.

4.3 Experimental design

4.3.1 Study cohort

To examine the relationships between HDL proteome and total and cardiovascular mortality, we studied participants of the Ludwigshafen Risk and Cardiovascular Health (LURIC) Study, a large and well defined cohort of patients. LURIC was an ongoing prospective study of environmental, biochemical, and genetic risk factors for CAD in a hospital-based cohort of white individuals. We studied patients who had undergone coronary angiography between June 1997 and January 2000, at the Ludwigshafen General Hospital and did not suffer from major non-cardiovascular disease. The study was approved by the institutional review board at the "Ärztekammer Rheinland-Pfalz". Informed written consent was obtained from each of the participants. All participants were profiled in detail with regard to established risk factors for cardiovascular disease. The cohort is followed for morbidity and mortality. The median follow up time is 8 years.

CAD was assessed by angiography using the maximum luminal narrowing estimated by visual analysis. In LURIC, clinically relevant CAD was defined as the occurrence of at least 1 stenosis 20% in at least 1 of 15 coronary segments. Samples were classified into cases and controls. Cases were defined as patients who died from cardiovascular causes and who may have had previous myocardial infarction (MI) or stroke (not within three months prior to recruitment). Controls were defined as patients who have not had any previous MI or stroke and did not develop any cardiovascular events during the entire follow-up study period.

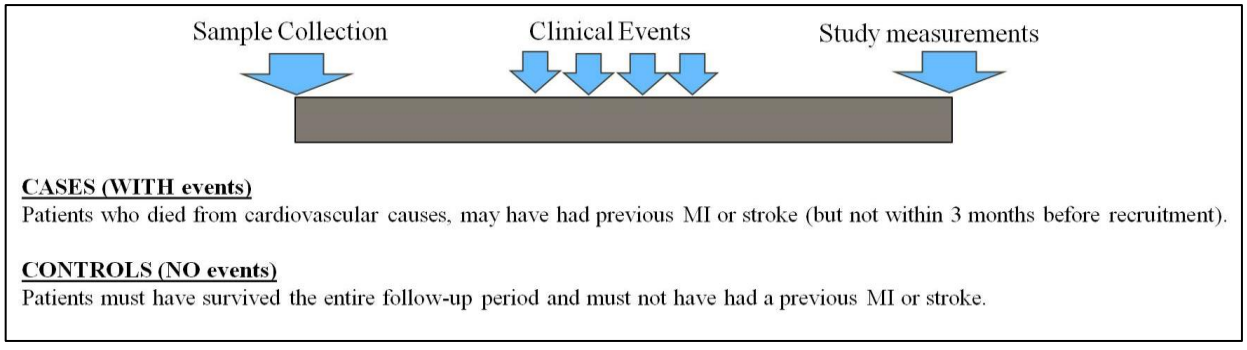


Figure 4.1 Illustration of the study design. Blood samples were collected at the start of the study upon patient recruitment. Patients who died during the course of follow-up due to cardiovascular causes were defined as cases. Patients who survived the entire follow-up, without developing any cardiovascular events, were classified as controls.

4.3.2 HDL isolation, delipidation and in-solution digestion

Serum was stored at −70 °C and thawed on 1 occasion for lipoprotein isolation. HDL was isolated as described previously by sequential ultracentrifugation (*d* = 1.063-1.21 g/ml) using solid potassium bromide (Merck KGaA, Darmstadt, Germany) for density adjustment.[24, 25] HDL was delipidated using methanol/chloroform extraction as previously described.[6] The protein pellet was dissolved in 50 mM ammonium bicarbonate at pH 8.0. Yeast protein, alcohol dehydrogenase (100ng/ml), was added as an internal standard control. Rapigest (0.1%) was added prior to reduction with 15 mM DTT for 30 minutes at 60ºC followed by alkylation of the cysteine residues with the addition of iodoacetamide to a final concentration of 40 mM for 45 minutes at room temperature and darkness. Enzymatic digestion was performed in 50 mM ammonium bicarbonate, pH 8.0 overnight at 37 ºC with 1 µg sequencing grade trypsin (Promega). Tryptic digests were desalted with silica C18 MacroSpin column (The Nest Group) and the peptides were dried in a speed vac concentrator and re-solubilized in 0.1% formic acid for mass spectrometric analysis.

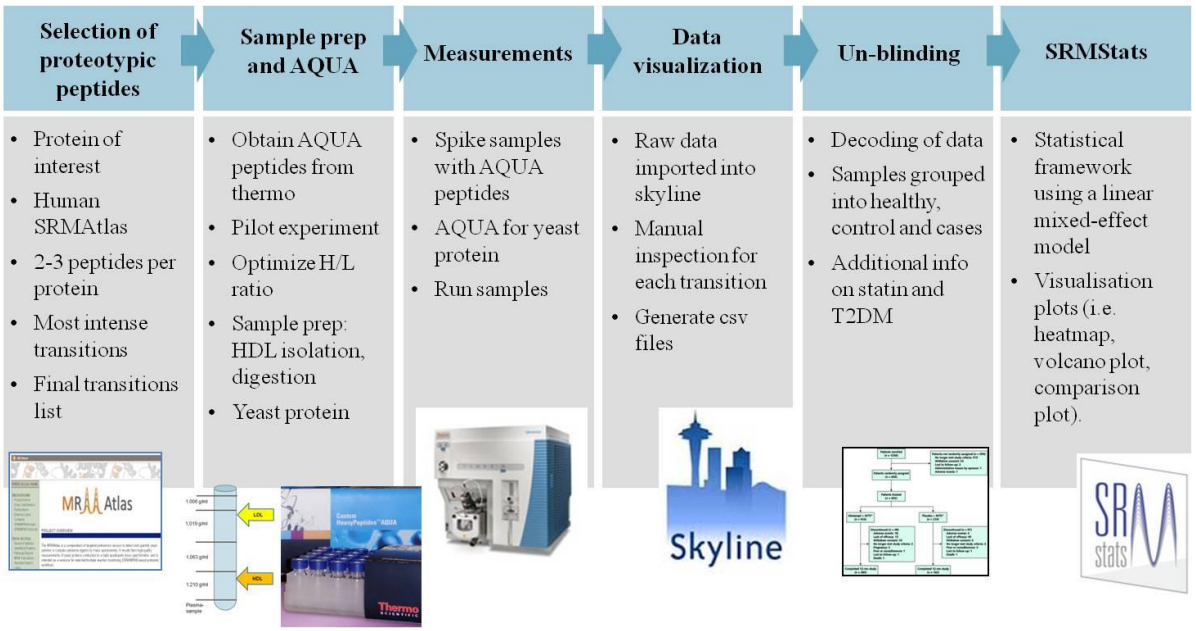


Figure 4.2 Diagram of the experimental workflow.

4.3.3 Generation of SRM assays

In contrast to conventional shotgun proteomic studies, SRM measurements are quantitative analyses strictly targeting a predetermined set of peptides and depend on specific SRM transitions for each targeted peptide. Previous information is required to define these transitions. Specifically, three types of information are of critical importance. First, the proteins that constitute the targeted protein set have to be selected. Second, for each targeted protein, those peptides that present good MS responses and uniquely

identify the targeted protein, or a specific isoform thereof, have to be identified. Such peptides have been termed as proteotypic peptides (PTPs).[26] Third, for each PTP, those fragment ions that provide optimal signal intensity and discriminate the targeted peptide from other species present in the sample have to be identified.

4.4 Results

4.4.1 Pilot experiment

Using SRM in combination with AQUA peptides, we first performed a pilot study of 10 cases and 10 controls to assess potential variability of the samples. We used skyline for the visualization of the acquired transitions and manual inspection of the signals were performed to correct for any discrepancies. The quantified transitions can be influenced by the technical MS run variability,e.g., due to changes in the instrument performance over a large number of MS runs. This technical variation can be removed by a global between-run normalization, e.g. by constant normalization that equalizes the median peak intensities of all reference transitions between runs. The normalization is based on the assumption that the intensities of the reference transitions are stable across all MS runs.

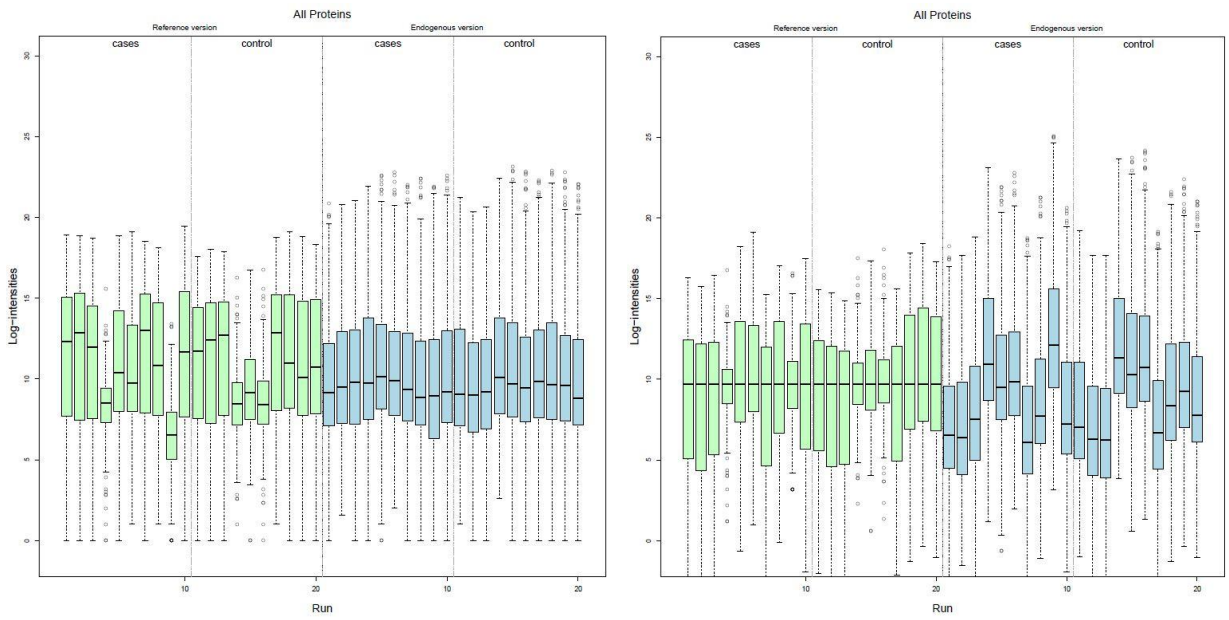


Figure 4.3 QC plots before (*Left*) and after (*Right*) normalization. QC plot illustrates the systematic bias between MS runs. After normalization, the reference signals for all proteins should be stable across MS runs. X-axis is run. Y-axis is log-intensities of transition. Reference/endogenous signals are in the left/right panel.

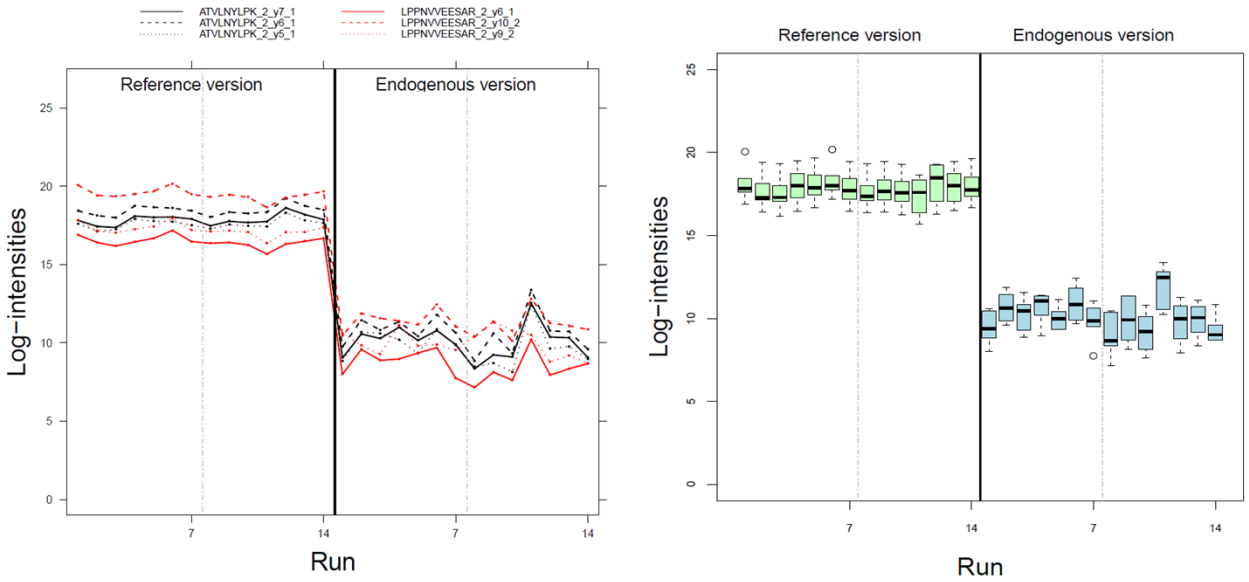


Figure 4.4 Quantified transition signals of a protein from each of the biological replicates, after a global normalization. Transitions of the reference peptides had a roughly constant abundance between runs. In contrast, transitions of the endogenous peptides indicated systematic differences between conditions, and these changes are of primary interest. The measurements were subject to biological variation of protein abundance and technical between-run variation. X-axis is run. Y-axis is log-intensities of transitions. Reference/endogenous signals are in the left/right panel. Line colors indicate peptides and line types indicate transitions.

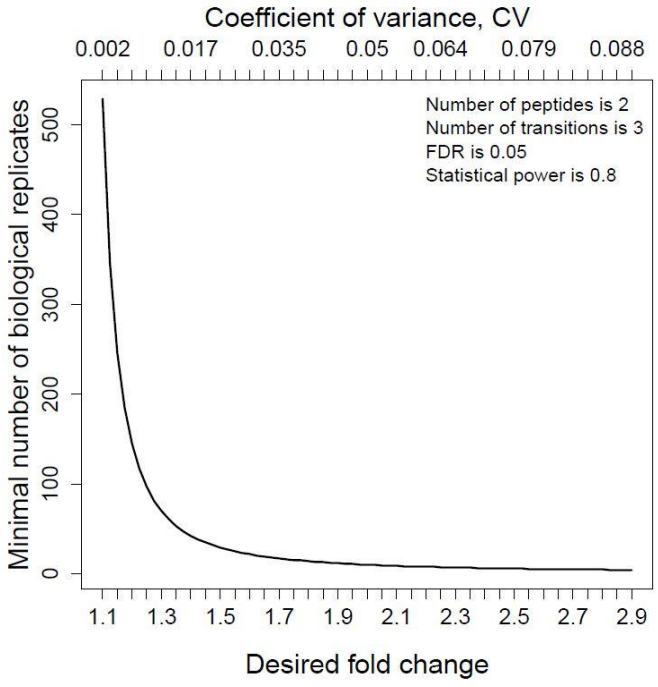


Figure 4.5 Illustration of the calculated minimal number samples size to obtain the statistical power based on the desired fold changes, the number of peptides per protein as well as number of transitions per peptide.

4.4.2 Quantification of candidate markers in HDL using SRM

We used SRM in combination with AQUA in order to detect and quantify the candidate proteins in HDL. We isolated HDL by sequential ultracentrifugation from serum samples of 61 cases, 70 controls and 7 healthy subjects. SRM assays were extracted from the SRMAtlas for 43 candidate HDL-associated proteins of interest identified in previous studies.[6, 8] Two to three proteotypic peptides were selected and corresponding C13 AQUA peptides were synthesized by Thermo Scientific and used as internal standards, allowing realiable quantification and accurate quantification of the peptides.[27] The resulting SRM data was visualized using skyline.[28] Statistical analysis was performed using a mixed effects model implemented in SRMstats.[29] Proteins were considered significantly up- or down- regulated with a p-value below 0.05 adjusted by Benjamini-Hochberg[30] and fold change larger than 1.2.

Interestingly, we observed 10 proteins that are significantly regulated between healthy subjects and control, and 15 proteins between healthy subjects and cases. These proteins are shown in Figure 4.6 and listed in Table 4.1. Some of these proteins have previously been reported to be differentially enriched in patients with CAD/ACS versus healthy control subjects, thereby further validating our experimental methods.

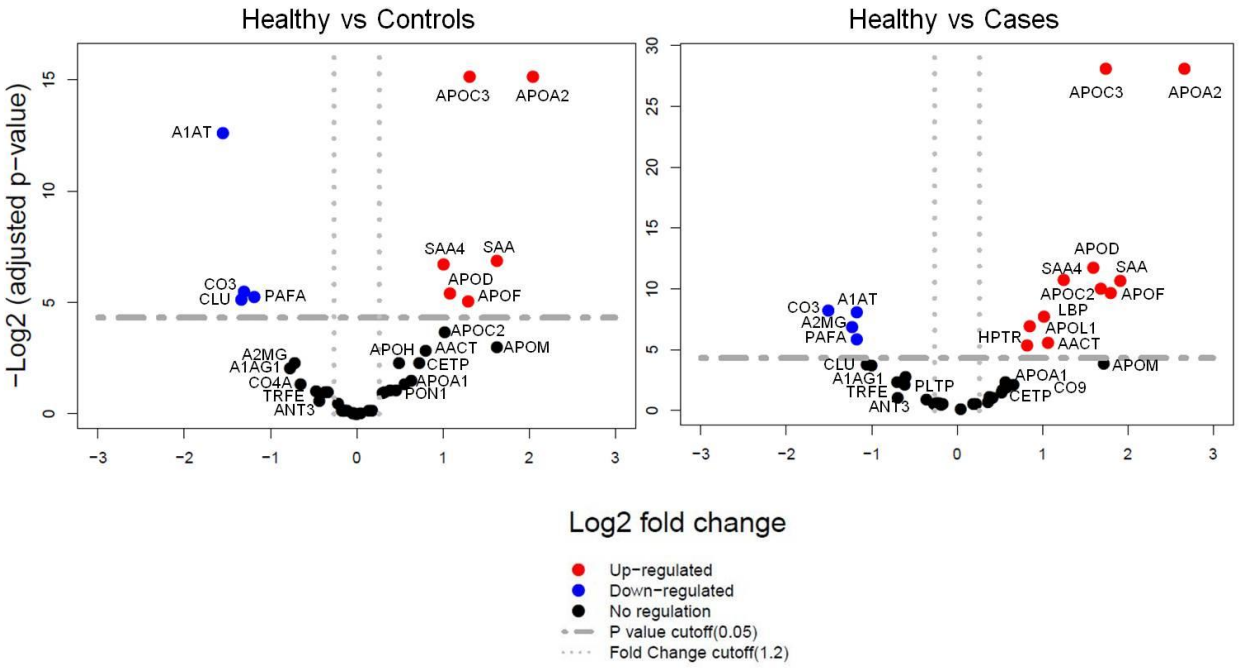


Figure 4.6 Volcano plots illustrating the results of the statistical analysis of HDL proteins from cases and controls versus healthy subjects. In the plots, proteins that are significantly down-regulated or up-regulated in HDL from cases or control as compared to healthy subjects are colored in blue or red, respectively.

Table 4.1 Proteins quantified by SRM that are significantly regulated in HDL from cases versus healthy and controls versus healthy. The fold change (log2FC), standard error (SE), T-value and adjusted p-value are given in the table.

Protein	Label	log2FC	SE	T-value	P-value
APOA2	healthy-control	2.045	0.412	4.970	2.76E-05
APOC3	healthy-control	1.311	0.269	4.877	2.76E-05
A1AT	healthy-control	-1.552	0.351	-4.416	1.60E-04
SAA	healthy-control	1.627	0.482	3.378	8.59E-03
SAA4	healthy-control	1.008	0.307	3.285	9.48E-03
CO3	healthy-control	-1.307	0.442	-2.958	2.21E-02
APOD	healthy-control	1.081	0.375	2.885	2.37E-02
PAFA	healthy-control	-1.190	0.424	-2.808	2.63E-02
CLUS	healthy-control	-1.339	0.488	-2.745	2.85E-02
APOF	healthy-control	1.294	0.482	2.687	3.03E-02
APOC3	healthy-cases	1.743	0.267	6.528	3.40E-09
APOA2	healthy-cases	2.665	0.409	6.516	3.40E-09
APOD	healthy-cases	1.595	0.372	4.282	2.90E-04
SAA4	healthy-cases	1.249	0.305	4.097	5.68E-04
APOF	healthy-cases	1.910	0.478	3.993	5.94E-04
APOC2	healthy-cases	1.682	0.439	3.835	9.61E-04
SAA	healthy-cases	1.799	0.479	3.759	1.23E-03
CO3	healthy-cases	-1.510	0.439	-3.439	3.23E-03
A1AT	healthy-cases	-1.177	0.349	-3.372	3.59E-03
LBP	healthy-cases	1.017	0.310	3.286	4.72E-03
APOL1	healthy-cases	0.850	0.276	3.082	7.99E-03
A2MG	healthy-cases	-1.231	0.406	-3.033	8.59E-03
PAFA	healthy-cases	-1.176	0.421	-2.794	1.69E-02
AACT	healthy-cases	1.066	0.393	2.714	2.07E-02
HPTR	healthy-cases	0.820	0.312	2.632	2.45E-02

We also observed some protein that are differentially regulated between cases and control (Figure 4.7, Table 4.2). Interestingly, the distribution of these proteins is narrower than those of healthy-cases and healthy-control. This suggests that the HDL proteome have been altered/remodeled even in the early stage of the disease (stable CAD). Some of these proteins are not differentially regulated between cases and control, suggesting that these proteins albeit may represent a good marker for the disease, has no value when identifying patients at risk of future events.

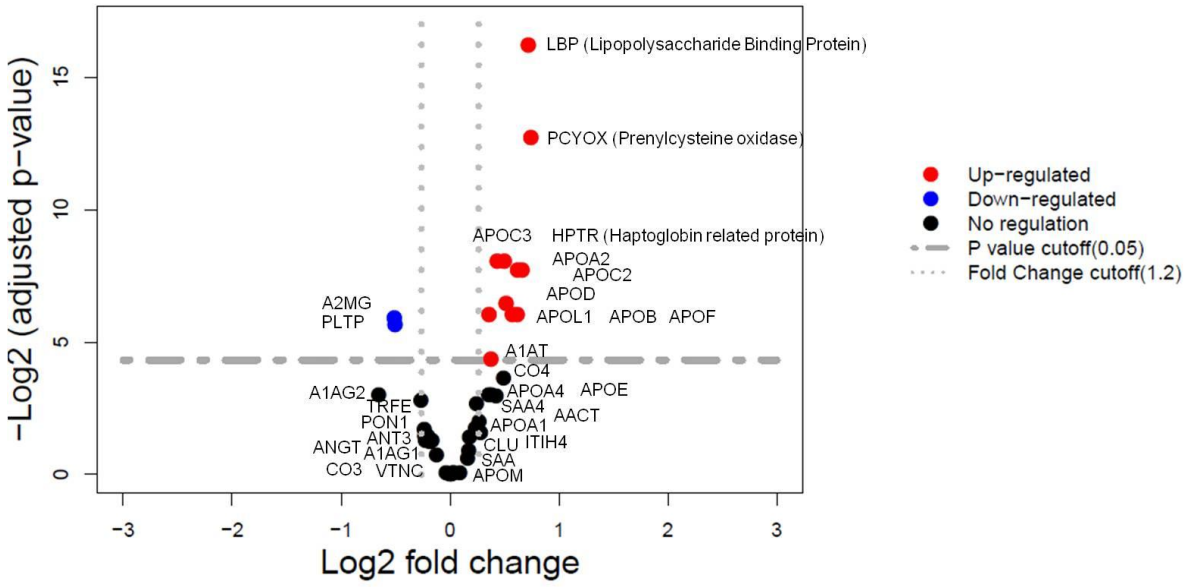


Figure 4.7 Volcano plot illustrating the results of the statistical analysis of HDL proteins from cases versus controls. In the plot, proteins that are significantly down-regulated or up-regulated in HDL from cases as compared to control are colored in blue or red, respectively.

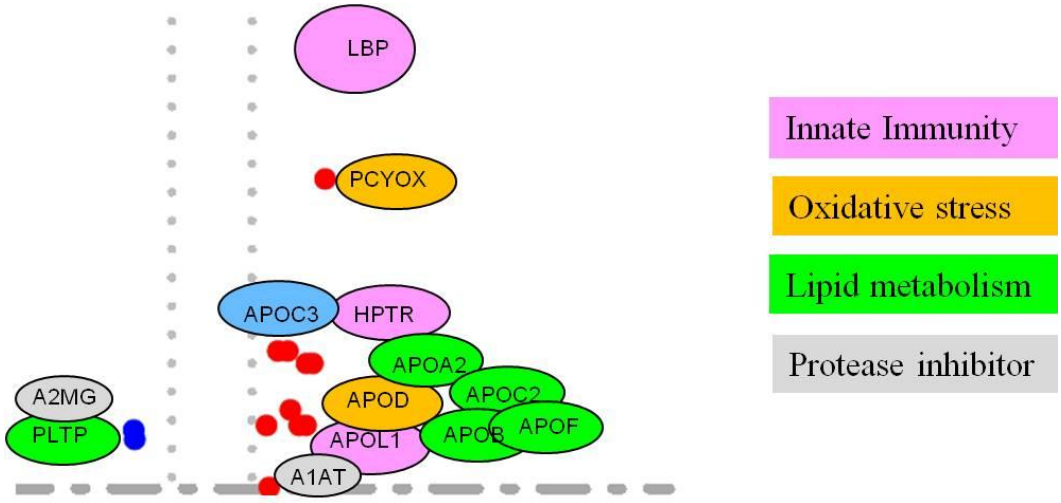
Table 4.2 Proteins quantified by SRM that are significantly regulated in HDL from cases versus controls. The fold change (log2FC), standard error (SE), T-value and adjusted p-value are given in the table.

Protein	Label	log2FC	SE	Tvalue	P-value
LBP	control-cases	0.718	0.137	5.246	1.29E-05
PCYOX	control-cases	0.743	0.162	4.582	1.45E-04
APOC3	control-cases	0.432	0.118	3.662	3.68E-03
HPTR	control-cases	0.498	0.138	3.612	3.71E-03
APOA2	control-cases	0.619	0.181	3.424	4.76E-03
APOC2	control-cases	0.662	0.194	3.411	4.76E-03
APOD	control-cases	0.514	0.165	3.112	1.14E-02
APOL1	control-cases	0.359	0.122	2.941	1.52E-02
APO1B	control-cases	0.571	0.195	2.925	1.52E-02
APOF	control-cases	0.616	0.212	2.912	1.52E-02
A2MG	control-cases	-0.512	0.180	-2.853	1.67E-02
PLTP	control-cases	-0.506	0.182	-2.777	1.94E-02
A1AT	control-cases	0.375	0.154	2.429	4.85E-02
CO4	control-cases	0.491	0.220	2.231	8.01E-02
APOA4	control-cases	0.378	0.192	1.966	1.22E-01
APOE	control-cases	0.355	0.181	1.965	1.22E-01

Notably, proteins which are significantly up/down-regulated between cases and controls can be classified into different biological processes (Figure 4.8). Besides the apolipoproteins and phospholipid transfer protein (PLTP) which are involved in lipid metabolism, other proteins are associated with innate immunity, oxidative stress, and protease inhibition. Apolipoprotein C-III (APOC3) inhibits lipoprotein lipase-mediated hydrolysis of triglycerides and its presence in HDL has been associated with incidence of coronary heart disease.[31] We recently showed that increased APOC3 content in HDL from patients with CAD activate the pro-apoptotic signaling in endothelial cells.[6] Lipopolysaccharide binding protein (LBP), haptoglobin related protein (HPTR) and apolipoprotein L1 (APOL1) have previously been described to play a role in the innate immune response.[32] LBP binds and neutralizes the bioactivity of the potent bacterial lipids, LPS and lipoteichoic acid, that stimulate host innate immune responses.[33] Depending on its concentration, LBP can either potentiate or inhibit responses to LPS.[34] Studies have also shown that HDL particles containing APOL1 and HPTR mediate the lysis of trypanosomes[35] and also inhibit Leishmania infection.[36] Prenylcysteine oxidase is an enzyme crucial for the degradation of prenylated proteins, generates free cysteine and hydrogen peroxide, suggesting a role in oxidative stress.[37] Apolipoprotein D is also known to be involved in aging and oxidative stress.[38, 39] Alpha-2-macroglobulin

(A2MG) has many diversified and complex functions, but it is primarily known by its ability to inhibit a broad spectrum of proteases.[40] Another protease inhibitor found to be differentially regulated between cases and controls is alpha-1-antitrypsin (A1AT) which is also an acute phase reactant.[41]

Figure 4.8 Classification of proteins identified into their associated biological functions and processes.



4.5 Discussion

Multiple prospective epidemiological studies support the importance of HDL cholesterol in atherosclerosis. However, the increase in HDL cholesterol observed with some lipid modifying drugs has not been uniformly associated with clinical benefit,[42, 43] indicating that the association between HDL and cardiovascular disease is more complex than previously suspected, and probably is mediated by different HDL functional properties, independent of the cholesterol component of the HDL particle.[44] A key challenge in understanding the physiology and pathophysiology of HDL in coronary disease is the heterogeneity of HDL particles, with different associated proteins modulating specific functions. One approach is to dissect the complexities of HDL by qualitative and quantitative evaluation of the proteins and lipids associated with HDL. And the next challenge is to understand how these changes may relate to clinical outcome.

Proteomics studies of HDL have been limited in number and size because of the labor-intensive nature of the present proteomics platforms. Due to the diverse concentration range of the proteins in the HDL particles, some proteomics approaches may fail to reflect the actual amount or alteration of low abundant proteins due to signal suppression in the presence of highly abundant proteins. Targeted proteomics using selected reaction monitoring is a powerful tandem mass spectrometry method that can be used to monitor

target peptides within a complex protein digest.[18, 19] The approach is highly specific and sensitive, and it also allows multiplexing measurement of many analytes in parallel.

In this study, we have used quantitative proteomics with SRM to evaluate the differences in the protein compositions of HDL isolated from patients with CAD who died from cardiovascular events versus patients with CAD who did not have cardiovascular events. HDL isolated from these patients has markedly altered protein compositions as compared to HDL from healthy subjects, which may account for the altered vascular effects of HDL in CAD shown in recent studies.

Differences in the protein compositions of HDL from patients who died from cardiovascular events versus patients who survived may identify potential markers in determining patients at risk of developing future cardiovascular events. An important finding from this study is that some of the proteins identified may allow better understanding of the mechanisms of action of HDL and its alterations in cardiovascular disease. Of note, some of the proteins identified to be differentially regulated are not related to lipoprotein metabolism, indicating that HDL physiology and its pathophysiology in CAD is beyond simply regulation of lipid metabolism but also other metabolic processes such as innate immunity and regulation of oxidative stress.

Nonetheless, the next key question is to understand whether these differences in the protein compositions can be translated into differences in HDL function. Furthermore, it is also crucial to evaluate whether the proteomic changes found in this study reflect a causal relationship or purely an association.

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Chapter 5

Discussion

Low serum concentration of HDL cholesterol is considered by many to be the strongest lipid risk factor for cardiovascular disease.[1] Clinical and epidemiological studies have shown a strong inverse association of plasma levels of HDL cholesterol with coronary heart disease.[2] Besides promoting macrophage cholesterol efflux and reverse cholesterol transport, HDL from healthy subjects has been shown to exert direct vasoprotective effects.[3-7] These have led to the development of the 'HDL cholesterol hypothesis', which suggests that intervention to raise HDL cholesterol will result in reduced risk of coronary heart disease. Consequently, considerable efforts have been devoted to the development of HDL cholesterol raising therapies.

However, several lines of evidence have raised serious doubts about the validity of the HDL cholesterol hypothesis. Recent clinical trials using the HDL cholesterol raising agents torcetrapib, dalcetrapib and niacin have shown that no significant reduction of cardiovascular events was observed in patients with coronary disease.[8-10] Moreover, recent mendelian randomization analyses showed that genetic variants associated with HDL cholesterol levels had a weak or nonexistent relationship with cardiovascular disease risk, particularly when they were only associated with HDL cholesterol levels and not with LDL cholesterol or triglyceride levels.[11] Taken together, these observations suggest that the association between HDL cholesterol levels and cardiovascular disease risk is not a causal relationship. HDL cholesterol may simply be a biomarker and elevating HDL cholesterol levels is not necessarily therapeutic.

We are beginning to appreciate that it is the quality and function of HDL, not the cholesterol levels, which may be mechanistically more important in relation to cardiovascular disease protection. Importantly, accumulating evidence suggests that the vascular effects of HDL can be highly heterogeneous and HDL may lose important anti-atherosclerotic properties and turn dysfunctional in patients with chronic inflammatory disorders.[12-21] Therefore, there is a great medical need for assays or markers that reflect the functionality of HDL better than HDL cholesterol levels. One approach towards this goal is to better understand the mechanisms of actions of HDL and its pathophysiology in cardiovascular diseases. Hitherto, the relative importance of many physiological and pathological roles of normal and dysfunctional HDL for the pathogenesis of atherosclerosis is unknown. The overall goal of this thesis was therefore to identify the components and biological activities of HDL that are most relevant for the protections against coronary artery disease.

The first pivotal finding of this thesis was that HDL isolated from patients with CAD, in comparison to HDL from healthy subjects, has altered effects on endothelial anti- and pro-apoptotic signaling pathways, leading to a loss of the endothelial anti-apoptotic capacity of HDL.[21] This study further demonstrates that reduced HDL-associated clusterin and increased HDL-associated apoC-III play an important role for altered activation of endothelial anti- and pro-apoptotic signaling pathways in HDL from patients with CAD. These observations raise the possibility that certain proteins in HDL play previously unsuspected

roles in regulating endothelial cell apoptosis. This action might be centrally important for preventing endothelial cell dysfunction and blocking multiple key steps in atherogenesis. Of note, it was observed that the signaling pathways activated by HDL in the regulation of endothelial apoptosis seem to be independent of its capacity to stimulate eNOS-mediated NO production, suggesting that different biological functions of HDL may be modulated independently by different HDL components, via specific signaling pathways. Whether certain pathological condition alters a particular component of HDL and whether this leads to specific impairment in biological function of HDL remains to be determined.

A major clinical challenge is to link molecular mechanisms underlying HDL's cardioprotective activities to robust, quantitative metrics that can be widely applied in translational and clinical studies. Reduced endothelial nitric oxide availability, pro-inflammmatory activation and endothelial cell apoptosis are regarded as key mechanisms in the development and progression of atherosclerosis. The capacity of HDL to modulate these endothelial effects may therefore represent a potentially important anti-atherogenic property of HDL. Furthermore, the effects of HDL on these endothelial functions have been shown to be regulated via specific signaling pathways and more importantly, these endothelial protective effects have to been shown to be impaired in coronary artery disease.[20, 21] Therefore, one potential approach for assessing HDL function is to measure the capacity of HDL to modulate these endothelial signaling pathways. In this regard, we have optimized cell-based assays to measure the capacity of HDL to inhibit TNF-α-induced VCAM-1 expression, to reduce active caspase-3 activation and to stimulate nitric oxide production.

We have used these assays to evaluate the impact of CETP inhibitor treatment on the endothelial effects of HDL. The first CETP inhibitor, torcetrapib, effectively increased HDL-C and lowered LDL-C but was associated with increased cardiovascular mortality.[8] Meanwhile, earlier-phase clinical trials have shown that dalcetrapib contribute to the reduction of atherosclerotic progression and vascular inflammation without adversely affecting blood pressure or aldesterone levels.[22, 23] However, despite these encouraging signals, the Phase III dal-OUTCOMES trial was terminated prematurely due to lack of clinically meaningful efficacy.[9] How do we reconcile our findings with the failure of CETP inhibitors in clinical trial? We observed that there was no difference in the improvement of HDL functions from baseline level when we compared placebo and dalcetrapib treatment groups. Furthermore, treatment with dalcetrapib failed to restore HDL functions to the level of healthy subjects, which may explain, at least in part, the lack of benefit on endothelial function seen in the dal-VESSEL trial.[24]

Interestingly, despite the failure of torcetrapib in clinical trial resulting in increased cardiovascular mortality, no impairment in HDL functions was observed following torcetrapib treatment in this study. This finding may explain the lack of improvement in the quality of HDL following torcetrapib treatment, however the adverse effects observed in the clinical trial of torcetrapib were likely caused by off-target

side effects, which may have been due to direct deleterious actions of torcetrapib on endothelial function,[25] independent of the HDL cholesterol raising effect.

Whereas the endothelial effects of HDL were not significantly altered following dalcetrapib or torcetrapib treatment, raising HDL cholesterol levels via these CETP inhibitors have not proven to be effective in reducing cardiovascular disease risk in clinical trials. These findings further support the notion that HDL cholesterol level is a noncausal biomarker. It seems prudent to re-evaluate the use of HDL cholesterol level as a biomarker predictive of cardiovascular disease risk in intervention studies.

Nevertheless, it is important to note that CETP inhibition may not provide a direct answer for testing the HDL hypothesis. A recent genetic study has shown that the *CETP* alleles associated with lower CETP activity, higher HDL cholesterol, lower LDL cholesterol, lower triglycerides, and lower lipoprotein(a) are indeed associated with decreased risk for cardiovascular disease,[26] suggesting that drugs that inhibit CETP activity are likely to reduce the risk for cardiovascular disease. While torcetrapib failure may be attributed to off-target side effects, dalcetrapib treatment primarily alters HDL cholesterol level with minimal effects on other lipid parameters.[9, 22] It is worth noting that large-scale outcome studies of anacetrapib and evacetrapib are ongoing. Anacetrapib and evacetrapib profoundly increase HDL cholesterol levels and also reduce LDL cholesterol levels by 35-40%,[27, 28] raising a distinct possibility that these CETP inhibitors will likely show benefit in reducing cardiovascular disease risk. However, the potential benefit from this CETP inhibition will likely be independent of their effects on HDL cholesterol elevation.

While recent events seem to have cast doubts on the HDL hypothesis, the anti-atherogenic role of HDL cannot be undermined and HDL remains an interesting target for treatment against cardiovascular diseases. A major challenge is to identify the components of HDL that are most relevant for the protections against coronary artery disease. HDL forms a heterogeneous class of lipoproteins which differ by protein and lipid compositions, some of which may contribute to the pleiotropic functions of HDL. Another pivotal finding of this thesis was that proteome remodeling in HDL has direct implications on functional properties, i.e. vascular effects of HDL. We observed that reduced HDL-associated clusterin and increased HDL-associated apoC-III may play a role for altered activation of endothelial apoptotic signaling pathways in HDL from patients with CAD. This further supports the notion that HDL dysfunction is associated with different structural changes in HDL including enrichment with acute phase proteins as well as protein modifications in pathological conditions.

There is a need to carefully dissect the complexities of HDL composition and function and identify relevant HDL components in order to generate important novel insights into the molecular pathophysiology of HDL-associated cardiovascular diseases. In this regard, we have developed and optimized quantitative proteomics analysis with selected reaction monitoring (SRM) to monitor alterations

in HDL-associated proteins in specific pathological conditions. The results of the analyses presented in this thesis are preliminary and work is currently ongoing to generate the full data sets of this targeted proteomics study. Nevertheless, based on our preliminary findings, we have observed that HDL from patients with CAD has significantly altered protein compositions as compared to HDL from healthy subjects, further supporting our earlier observations. Notably, significant differences were observed in the HDL proteome between patients with CAD who died from cardiovascular events versus patients who did not develop any events during the entire follow-up study period. This finding may identify potential markers in determining patients at risk of developing future cardiovascular events. More importantly, some of the proteins identified will allow better understanding of the mechanisms of action of HDL and its alterations in cardiovascular disease. Of note, some of the proteins identified to be differentially regulated are not related to lipoprotein metabolism, indicating that HDL physiology and its pathophysiology in CAD is beyond simply regulation of lipid metabolism but also other metabolic processes such as innate immunity and regulation of oxidative stress. Furthermore, quantifying the HDL proteome may provide insights into the therapeutic efficacy of antiatherosclerotic interventions.

In conclusion, I believe this thesis has contributed a step closer to understanding HDL physiology and its pathophysiology in coronary artery disease, in particular the endothelial effects of HDL. The proteomics study has yielded several novel HDL-associated protein candidates that may have played previously unsuspected roles in the pathogenesis of HDL-associated diseases. Nonetheless, in order to successfully exploit HDL as a treatment target in cardiovascular diseases, much remains to be done. Future work is needed to understand whether alterations in the HDL proteome are causal or simply an association. Furthermore, it is important to investigate how these HDL-associated proteins affect their cellular interaction partners and downstream effectors. Last but not the least, the complexity of HDL is further intensified with the presence of associate lipids and micro-RNAs. The contribution of these components towards HDL functionality will need to be explored. These important studies will generate important novel insights into the complexities of HDL and the molecular pathophysiology of cardiovascular disease. Importantly, this knowledge will drive the discovery and development of novel HDL-targeted therapies against cardiovascular diseases.

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Curriculum Vitae

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Work experience

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